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1. REPORT DATE (DD-MM-YYYY) 20-12-2002	2. REPORT TYPE Thesis	3. DATES COVERED (From - To) Jan 01 - Dec 02		
4. TITLE AND SUBTITLE <i>Improving the Efficiency of Enzymatic Baeyer-Villiger Oxidations with whole Engineered <u>Escherichia coli</u> Cells</i>		5a. CONTRACT NUMBER		
		5b. GRANT NUMBER		
		5c. PROGRAM ELEMENT NUMBER		
		5d. PROJECT NUMBER		
		5e. TASK NUMBER		
		5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) performed at:  CPT Adam Z. Walton c/o General Delivery West Point, NY 10996		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)  Advanced Civil Schooling Program Commander, PERSCOM ATTN: TAPC-OPB-D 200 Stovall St. Alexandria, VA 22332-0411		10. SPONSOR/MONITOR'S ACRONYM(S)		
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION/AVAILABILITY STATEMENT Unrestricted distribution; approved for public release				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT <i>See pg. viii</i>				
15. SUBJECT TERMS				
16. SECURITY CLASSIFICATION OF: a. REPORT <input checked="" type="checkbox"/> b. ABSTRACT <input checked="" type="checkbox"/> c. THIS PAGE <input checked="" type="checkbox"/>		17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES 71	19a. NAME OF RESPONSIBLE PERSON CPT Adam Walton
				19b. TELEPHONE NUMBER (Include area code) (352) 870-5940

Standard Form 298 (Rev. 8/98)  
Prescribed by ANSI Std. Z39-18

20021231 042

IMPROVING THE EFFICIENCY OF ENZYMATIC BAEYER-VILLIGER  
OXIDATIONS WITH WHOLE ENGINEERED *Escherichia coli* CELLS

By

ADAM ZANE WALTON

A THESIS PRESENTED TO THE GRADUATE SCHOOL  
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2002

This work is dedicated to my sons, Cameron, Logan and Ethan

## ACKNOWLEDGMENTS

I would like to thank my wife, Jennifer, and our boys for their unconditional patience and support during the past two years. This is not the first time I have requested such sacrifices, nor, apologetically, is it the last. To my grandfather, Harold A. Walton, who passed away during my pursuit of this work, I thank him for the encouragement and guidance that brought me to undertake graduate study. From the skies over Normandy to a career as a small town educator he is an icon of America's "greatest generation" and my inspiration. Acknowledgements are given to the U.S. Army and the Advanced Civil Schooling Program for its financial support and giving me time away from the job I love to develop myself professionally. I need to extend special thanks to Dr. Tomas Hudlicky for the generous loan of the Braun Biostat E and accessories, without which this work would not have been possible. I thank all the members of the Stewart Group, for the unique contribution each member has made to the progress of this research. And finally, I thank Dr. Jon Stewart for all the patience, advice and guidance that has yielded this successful work and contributed to my personal betterment.

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Abstract of Thesis Presented to the Graduate School  
of the University of Florida in Partial Fulfillment of the  
Requirements for the Degree of Master of Science

IMPROVING THE EFFICIENCY OF ENZYMATIC BAEYER-VILLIGER  
OXIDATIONS WITH WHOLE ENGINEERED ESCHERICHIA COLI CELLS

By

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December 2002

Chair: Jon D. Stewart

Major Department: Chemistry

Enzymatic Baeyer-Villiger oxidations are an extremely powerful tool in the synthesis of chiral lactones. Unfortunately, when purified enzymes and cofactors are involved, these methods are burdened with high costs and require significant expertise to isolate and maintain enzyme activity. Whole cells expressing the desired enzyme, in this case *Acinetobacter* sp. NCIB 9871 cyclohexanone monooxygenase (CHMO), answer the drawbacks associated with isolated enzyme catalysis but suffer from long reaction times and generally low product titers (often around 10 mmol/L).

We have developed methodology to conduct these whole cell Baeyer-Villiger oxidations with both growing and non-growing cells with nearly an order of magnitude greater product titers (80-90 mmol/L). These results are comparable to other published whole cell biocatalyst productivities. We believe these improvements make whole cell catalysis much more relevant for commercial applications.

As part of these studies, we have investigated the factors that potentially limit the productivities of whole cell oxidations. The required NADPH co-factor was supplied by an efficient and inexpensive carbon source, in our case glucose. This is critical in maximizing volumetric productivity. However, even with this improvement, reactions with whole cells expressing CHMO are limited to *ca.* a 24 hour time period. This is most likely due to enzyme loss as a result of the natural instability of the enzyme under operational conditions. Improvements to this methodology will require selection of alternate enzymes that display a greater stability profile while maintaining our currently obtained productivity.

## CHAPTER 1 BACKGROUND

The aim of this work is to develop methodology for conducting whole cell biocatalysis that greatly improves its productivity and therefore overcomes the primary disadvantages associated with this technique: long reaction times and low product titers. The model system used for this work is a recombinant *Escherichia coli* strain that over expresses the enzyme cyclohexanone monooxygenase (CHMO), originally isolated from *Acinetobacter* sp. NCIB 9871 (Chen *et al.*, 1999). We believe that the results achieved here may drastically enhance the potential commercial viability of this enzyme as well as many other whole cell biocatalysis applications.

### **Baeyer-Villiger Oxidation Methods**

Baeyer-Villiger oxidations have been a powerful and well studied tool for the synthetic chemist for over 100 years (Baeyer and Villiger, 1899). They enable direct conversion of ketones to esters with predictable insertion of oxygen. Methods for this type of reaction include traditional “chemical” methods (Krow, 1993; Renz and Meunier, 1999) and an emerging array of enzymatic techniques (Stewart, 1998).

### **Chemical Oxidations**

Chemical conversion of ketones to esters can be accomplished using an organic peracid or similar compound as the oxidizing agent. The same mechanistic framework established by Criegee (Criegee, 1948) appears to hold for all Baeyer-Villiger oxidations, whether carried out “chemically or “biologically.” Nucleophilic attack of the peracid terminal oxygen at the ketone carbonyl carbon is followed by migration of the adjacent

carbon-carbon bond along with simultaneous cleavage of the oxygen-oxygen bond to yield the ester product and the oxidant by-product (Figure 1-1). When the  $\alpha$ -carbons of the starting ketone are not equivalent, the most substituted, and therefore most stable carbocation-like carbon, will preferentially migrate (Vollhardt, 1987).

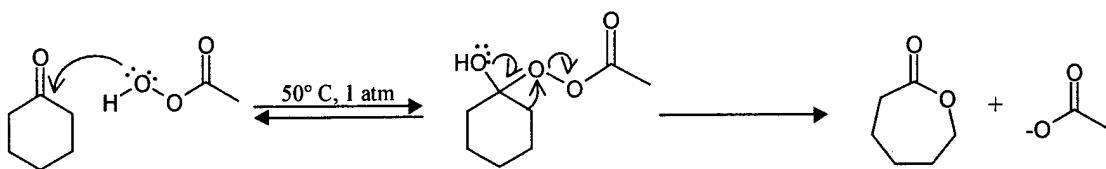


Figure 1-1. “Chemical” mechanism for the synthesis of  $\epsilon$ -caprolactone.  $\epsilon$ -Caprolactone is currently manufactured by this process for use in poly-esters and can be converted to  $\epsilon$ -caprolactam for production of nylon 6 (Weissermel and Arpe, 1997).

### Enzymatic Oxidations

The ability to perform enzymatic Baeyer-Villiger oxidations on cyclic ketones emerged on the scientific scene in the early 1970’s (Griffin and Trudgill, 1972; Donoghue and Trudgill, 1975). What started as a curiosity among microbiologists has evolved into useful methodology for asymmetric synthesis. *Acinetobacter* sp. NCIB 9871 cyclohexanone monooxygenase was first discovered as catalyzing an intermediate step in the metabolism of cyclohexanol (Figure 1-2) (Donoghue and Trudgill, 1975), and most of the information on this class of enzyme has been derived from this catalyst.

Since its discovery, it has become apparent that *Acinetobacter* CHMO is one member of a growing family of enzymes known as Baeyer-Villiger monooxygenases (BVMO). These include enzymes characterized by their ability to oxidize cyclohexanone (Brzostowicz *et al.*, 2000; Cheng *et al.*, 2000; Donoghue and Trudgill, 1976; Trower *et al.*, 1985), cyclopentanone (Griffin and Trudgill, 1972), camphor (Trudgill *et al.*, 1966),

testosterone (Morii *et al.*, 1999), dihydrocarvone (Van der Werf, 2000), and cyclododecanone (Kostichka *et al.*, 2001; Schumacher and Fakoussa, 1999).

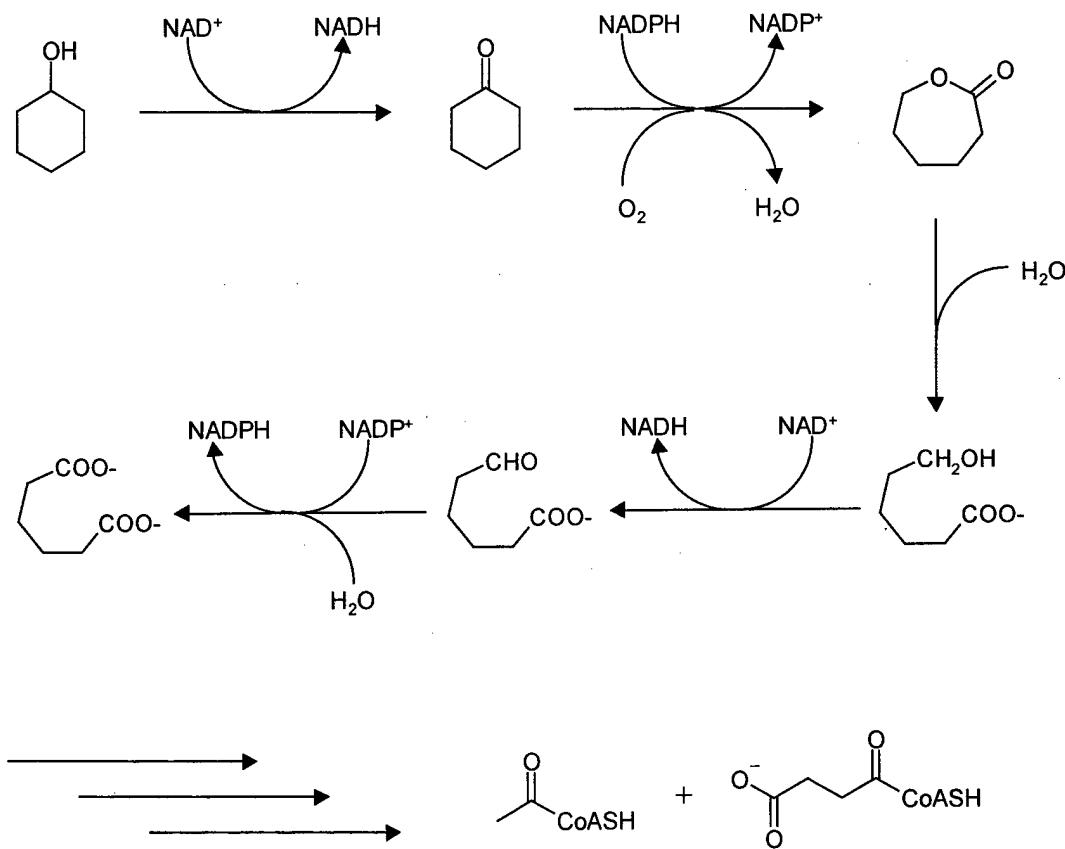


Figure 1-2. Metabolism of cyclohexanol in *Acinetobacter* sp. NCIB 9871 (Donoghue and Trudgill, 1975).

*Acinetobacter* NCIB 9871 CHMO was also the first enzyme of the Baeyer-Villiger class of monooxygenases to be cloned and sequenced (Chen *et al.*, 1988). Access to the cloned gene has allowed the construction of several recombinant overexpression systems (Stewart *et al.*, 1996a; Chen *et al.*, 1999; Cheesman *et al.*, 2001; Doig *et al.*, 2001) that have significantly expanded the utility of this enzyme and made it a model for the entire family.

The sequence of *Acinetobacter* CHMO has also been used to identify potential new Baeyer-Villiger monooxygenases. Recent advances in gene cloning technology have allowed the isolation of entire gene clusters from other organisms responsible for the metabolism of a variety of ketones (Brzostowicz *et al.*, 2002; Cheng *et al.*, 2000; Iwaki *et al.*, 1999; Kostichka *et al.*, 2001). BLAST searches of completed genome sequences have also yielded uncharacterized BVMO homologs with high sequence similarity to *Acinetobacter* CHMO in *Caulobacter crescentus*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*. Homologs among these species often occur in pairs, which has also been observed in other organisms (Brzostowicz *et al.*, 2000; Norris and Trudgill, 1973; Van der Werf, 2000).

While the focus of this work has been on the exploitation of *Acinetobacter* CHMO, our methodology is general, and could one day be applied to these newly-discovered BVMOs, thereby increasing their utility in organic synthesis.

#### ***Acinetobacter* sp. CHMO Substrate Specificity**

Synthetic chemists have been drawn to CHMO as a means of conducting asymmetric synthesis of chiral lactones. These oxidations have been thoroughly reviewed (Stewart, 1998) and expanded upon in recent years (Kayser *et al.*, 1998; Mihovilovic *et al.*, 2001a, 2001b). Examples that demonstrate this enzyme's selectivity are pictured in Figure 1-3.

Analysis of all known cyclohexanone substrate stereoselectivities has allowed for the formulation of a diamond lattice model for allowed substrate structures (Figure 1-4, Stewart, 1998). This model has been particularly useful in predicting potential substrates for this enzyme, since no X-ray crystal structure is available.

The natural substrate for *Acinetobacter* sp. CHMO is not known. By contrast, the situation is clearer for *Rhodococcus erythropolis* DCL14, which is able to utilize carveol and limonene as its sole carbon and energy sources. Intermediates in this metabolic pathway are the isomers of dihydrocarvone (Figure 1-5) that undergo a Baeyer-Villiger oxidation by one of two different monooxygenases before ring cleavage and subsequent metabolism (Van der Werf, 2000). Interestingly one of these two enzymes displays the opposite stereoselectivity as that of *Acinetobacter* CHMO (Alphand and Furstoss, 1992b). The ability to metabolize this class of mono-terpenes present in plant decay matter (also found in the source of *Acinetobacter* NCIB 9871) point directly to this as being the natural substrate for CHMO rather than non-naturally occurring cyclohexanone.

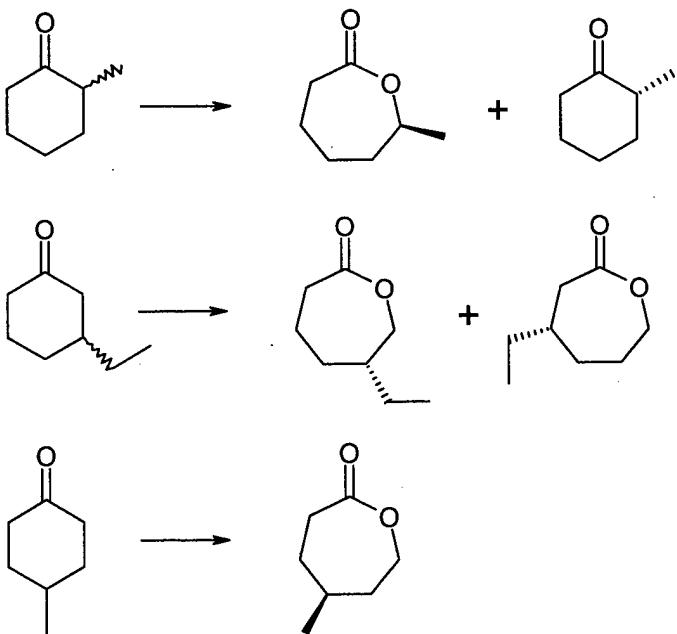


Figure 1-3. Examples of CHMO stereoselectivity.

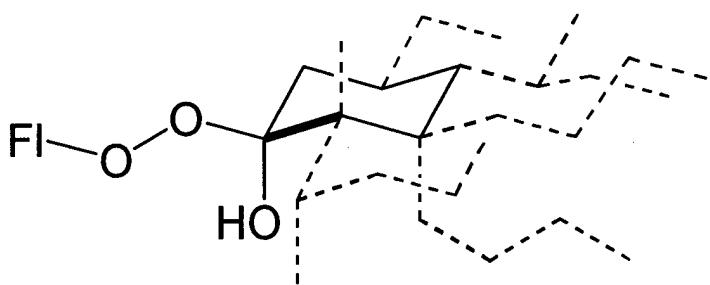


Figure 1-4. Lattice model for allowed substrate conformations (Stewart, 1998). Migrating carbon-carbon bond is indicated in heavy line. Allowed conformers of substitutions are depicted in dashed line.

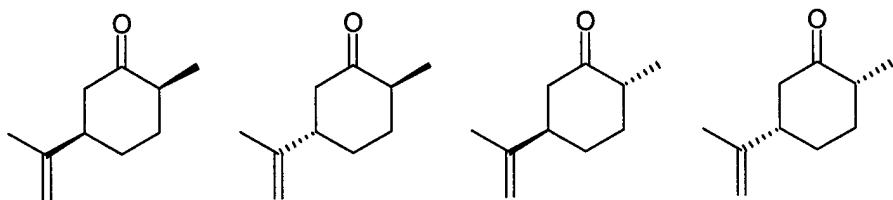


Figure 1-5. Naturally occurring isomers of dihydrocarvone.

### Kinetic Behavior

The overall reaction for CHMO oxidation of cyclic ketones is a *ter-ter* ordered mechanism (Ryerson *et al.*, 1982; Figure 1-6). The catalytic cycle begins with binding of NADPH, which then reduces the tightly bound ( $K_D$  40 nM) FAD co-factor. Oxygen then reacts to yield a C(4a)-peroxide intermediate. Subsequent nucleophilic attack on the carbonyl carbon of the ketone substrate by the terminal peroxide oxygen followed by bond migration follows the traditional Baeyer-Villiger scheme via the Criegee adduct. Flavin dehydration, lactone dissociation, and release of oxidized NADP follow to yield the inactive FAD<sub>ox</sub> bound enzyme. The rate determining step has been shown to be release of NADP<sup>+</sup> from the enzyme (Sheng *et al.*, 2001).

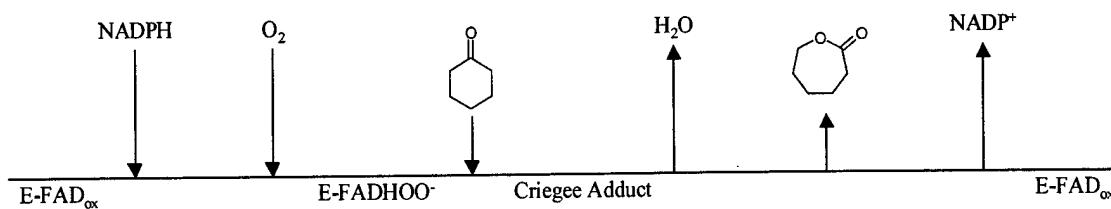


Figure 1-6. Kinetic mechanism of CHMO.

### Applications of *Acinetobacter* CHMO

#### Isolated Enzyme Catalysis

Reactions carried out with purified enzyme allow for the greatest control over the synthetic reaction; however, they also have several drawbacks. Foremost is the requirement to provide the NADPH co-factor. Not only is the co-factor expensive, it often demands the use of additional enzymes to “recycle” the co-factor to the reduced form if sub-stoichiometric quantities of NADPH are employed for economic reasons. In addition to these co-factor issues, enzyme purification is costly and time consuming and this is a second disadvantage of using the isolated monooxygenase. Even in the case of histidine tagged CHMO produced in recombinant *E. coli* (Cheesman *et al.*, 2001), the single step purification still requires specialized knowledge, equipment and time.

Many examples of isolated enzyme catalysis have been focused on producing small quantities and titers of product (~ 10 mmoles/L) for a broad range of substrates in order to both characterize the regio- and stereoselectivity of CHMO and perform a variety of small-scale synthesis (Taschner and Black, 1988; Abril *et al.*, 1989; Taschner and Peddada, 1992; Taschner *et al.*, 1993). These examples utilized glucose-6-phosphate dehydrogenase and its extremely expensive substrate, glucose-6-phosphate, to recycle the NADPH co-factor.

More recently, several groups have expanded practical methods to scale up these oxidations, emphasizing reduced cost and improved productivity. One example is the oxidation of 4-methyl cyclohexanone using formate dehydrogenase (FDH), derived from multiple mutations of the gene found in *Pseudomonas* sp. 101, and sodium formate to recycle NADPH (Rissom *et al.*, 1997). Ultrafiltration was used to contain the two enzymes and this method produced an effective yield of 120 mM lactone, with an average rate of 4.0 mM/hr over the time that the reactor was in use. Maximal rates ran at approximately 83% of  $V_{max}$  for this substrate, presumably due to limitations in delivery of the oxygen substrate feed. The batch processing method used here also used a reduced substrate concentration at later times, which thereby reduced the average hourly rate of lactone production.

Oxidation of racemic bicyclo[3.2.0]hept-2-en-6-one into two lactone regio-isomers provides another example of an improved process using purified CHMO (Figure 1-7) (Zambianchi *et al.*, 2002). NADPH recycling was accomplished by the use of alcohol dehydrogenase from *Thermoanerobium brockii* (ADHTB) and the very inexpensive substrate 2-propanol. The best results were obtained with a continuous ketone substrate feed and reached a total yield of 92 mM, with an average rate of 3.8 mM/hr. Initial rates ran at approximately 67% of the estimated  $V_{max}$  for this substrate, presumably due to substrate inhibition ( $K_i$  0.12 mM) and remained effectively constant over the course of the reaction.

Based on these examples, current oxidations of CHMO using isolated enzymes yield on the order of 0.1 M product in a 24-hour time period. The time and resources required for acquisition of pure enzymes (CHMO, FDH, ADHTB, G6PDH) and

substrates ( $\text{NADP}^+$ , G-6-P) must be weighed against the value of the lactone product for these methods to become industrially relevant.

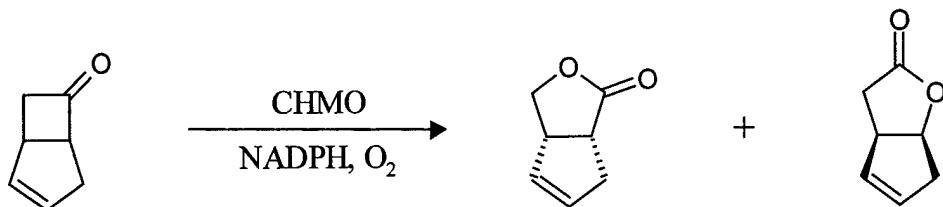


Figure 1-7. Reaction of bicycloheptenone with CHMO. Product lactones are useful in the synthesis of prostaglandins and nucleosides (Zambianchi *et al.*, 2000)

### Whole Cell Biocatalysis

The majority of whole cell biocatalysis examples using *Acinetobacter* CHMO have been saddled with one major drawback – low product titers. This phenomenon is found in biotransformations done with whole *Acinetobacter*, done largely by Furstoss *et al.* (Alphand *et al.*, 1990, 1996; Alphand and Furstoss, 1992a, 1992b), recombinant *Saccharomyces cerevisiae* (Kayser *et al.*, 1998; Reed, 1996; Stewart *et al.*, 1996a, 1996b, 1998) and *E. coli* (Chen *et al.*, 1999; Mihovilovic *et al.*, 2001a, 2001b) systems. Typical final product concentrations are on the order of 10 mM and, like their purified enzyme counterparts, these reactions have only been useful in the rapid, accurate, and inexpensive characterization of CHMO substrate and stereoselectivity.

On the other hand, whole cell biocatalysis is cheap. Enzyme production and co-factor recycling occur inside the host cell providing a self-sustaining and regenerating catalytic machine. Costs are mainly associated with the media formulations required for preparation of cells. Experience in enzymology or microbiology are not required, making it a method accessible to the average synthetic chemist.

Improving the productivity beyond observed limits of whole cell systems has only been addressed very recently. One example, portions of which have already been

published (Walton and Stewart, 2002), is covered in this writing. Another recent example, disclosed during this investigation, involves oxidation of the previously described bicycloheptenone (Figure 1-7; Simpson *et al.*, 2001). In the example of Simpson *et al.*, induced and subsequently harvested cells were suspended in non-growth medium prior to addition of substrate. Glycerol was used as the carbon source and the substrate was added via a saturated hydrophobic adsorption resin. Product yield prior to purification was 154 mM, with an average hourly yield of 3.4 mM/hr.

### **Long-term objectives**

In order for whole cell Baeyer-Villiger oxidations to become competitive with other methodologies, they must provide for final product levels of ~100 mM and rates of  $\geq 4$  mM/hr, on a wide variety of substrates. The aim of this work is to develop simple methodology to expand the applicability of whole cells expressing *Acinetobacter* sp. CHMO from a characterization technique to a tool capable of producing chiral lactones at a scale relevant to the chemical industry and the sophistication of an operation conducted by a one-armed man in a bathtub\*. It is our hope that the whole cell methodology developed here will also be applicable to a wider class of NADPH dependent enzymatic reactions.

\*The ideal bioprocess has been defined as a reaction run by a one-armed man in an open bathtub, who isolates pure product in quantitative yield by opening the drain and collecting it in a bucket.

## CHAPTER 2 RESULTS

Work done towards our goal of improving whole cell methodology can be classified into two major areas, 1) improving biotransformation yields and 2) identifying factors that limit these yields. In the course of these investigations we have also developed a novel method for isolation of our final product. Results in each of these areas will be discussed in this chapter.

### **Biotransformations**

The conversion of cyclohexanone to  $\epsilon$ -caprolactone with *Acinetobacter* CHMO is the model system used for this work. Cyclohexanone was chosen because it is relatively inexpensive and a good substrate for the chosen enzyme. Substrate cost was critical given that under high aeration conditions, loss of up to 25% of the added cyclohexanone was observed as a result of evaporation. While the product is also low-priced, it is the highest volume lactone synthesized by industry today (Thomas *et al.*, 2002). It is unlikely that enzymatic production of  $\epsilon$ -caprolactone will displace chemical methods around which the current industry infrastructure is built; however, optimization with this simple substrate may allow for efficient production of other, higher value products.

In all cases examined in this study, the Baeyer-Villiger oxidation was done with the *Escherichia coli* strain BL21(DE3) containing the plasmid pMM4 for overexpression of CHMO (Chen *et al.*, 1999). The plasmid contains genes for ampicillin resistance and CHMO. Expression of the latter is under the control of the phage T7 RNA polymerase. When induced with isopropyl- $\beta$ -thioglucopyranoside (IPTG), the efficient T7 polymerase

allows for total CHMO protein production that equals nearly 20% of the total cellular protein (Chen *et al.*, 1999).

Unless otherwise noted, all biotransformations were conducted in 3 liter working volumes with a Biostat E (B. Braun Biotech) bio-reactor.

### Biotransformation in Complex Media

Biotransformations in complex, or non-defined, medium were accomplished by culturing BL21(DE3)(pMM4) in Luria-Bertani (LB) medium (Figure 2-1). CHMO production was induced with IPTG in early log phase followed by addition of ketone substrate a short time later (15-20 minutes). Conversion to the oxidized product was then followed by gas chromatography.

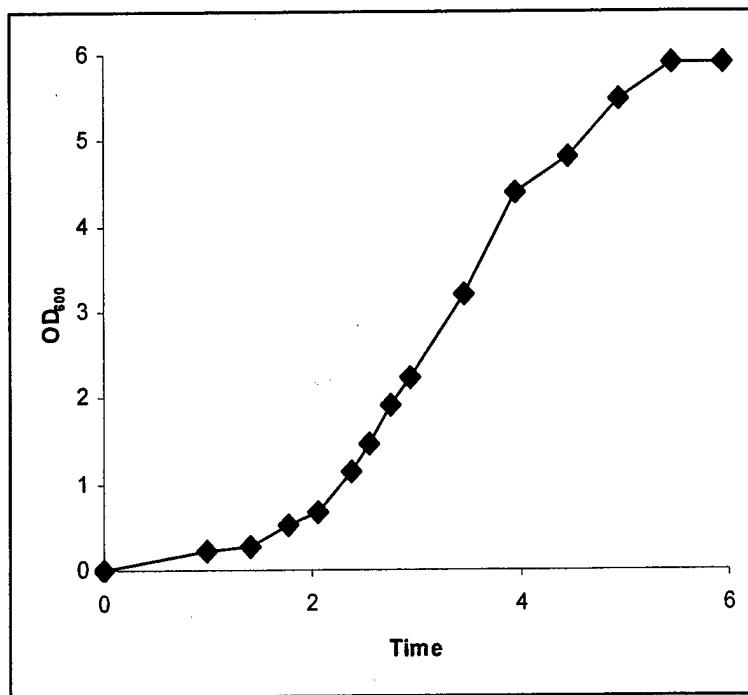


Figure 2-1. Standard growth curve for BL21(DE3)(pMM4) in LB media. Biomass concentration is expressed as optical density of culture medium at 600 nm.

The time at which the culture was induced directly affected the rate of conversion (Figure 2-2). The later in the growth cycle that IPTG was added, the faster the rate of

cyclohexanone oxidation. The maximum final product level of this system was 10 mM. That is, quantities of substrate added above 10 mM were not converted. This applied both to substrate bolus additions and reactions slow-fed cyclohexanone via pump. This 10 mM final product titer "wall" was the first hurdle we set out to overcome. It should be noted that the reaction that was induced at an optical density of 0.5, depicted in Figure 2-2, failed to come to 100% conversion by the time the growth reached stationary phase. This was the primary reason that we assumed that catalysis would not take place once cells had completed the logarithmic phase of growth. As will be seen, this was an oversimplification.

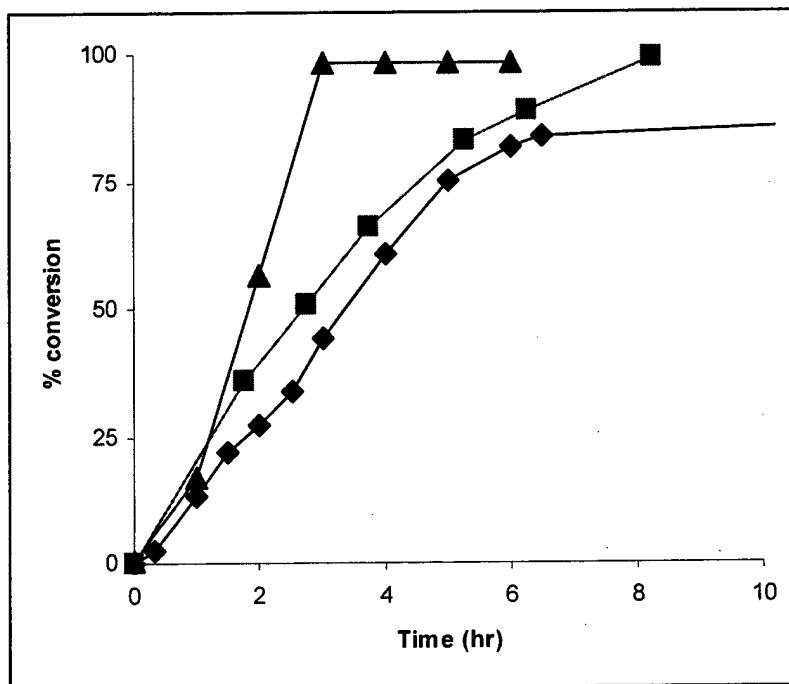


Figure 2-2. Conversion of 10 mM cyclohexanone at varied induction times by growing BL21(DE3)(pMM4) cells. IPTG was added to a final concentration of 0.1 mM at  $OD_{600} = 0.5$  (◆), 1.0 (■), and 2.0 (▲).

Under these growth conditions, significant quantities of CHMO were observed by SDS-PAGE within 15 minutes of induction (Figure 2-3). It is presumed that the

increased reaction rate was a direct result of the larger quantity of cells present and therefore the larger concentration of enzyme available for catalysis.

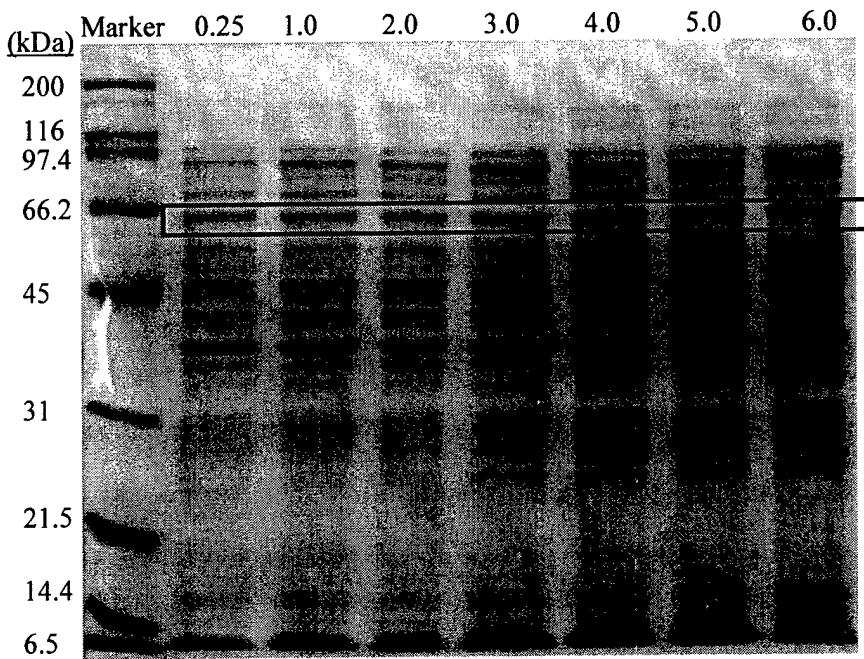


Figure 2-3. SDS-PAGE gel of IPTG induced cells grown in LB. CHMO band (~61 kDa) is indicated in lined box. Time post-induction in hours is indicated above each lane.

#### Biotransformations in Minimal Media

In order to better understand and control the variables that appeared to limit catalysis to actively dividing cells, we chose to analyze this biotransformation in a simple and defined growth medium. We chose M9 minimal medium (Sambrook *et al.*, 1989), which contains 60 mM phosphate buffered to pH 7.0, with glucose as a carbon source and ammonium chloride as a nitrogen source, supplemented with small amounts of magnesium and calcium. Growth in this medium was very slow and a low biomass yield was obtained (Figure 2-4) compared to LB media.

Using our traditional protocol we chose to induce this reaction at an  $OD_{600}$  of 1.0 in order to give large enough quantities of biomass for efficient catalysis and still leave enough time (~10 hours) before reaching the “terminal” stationary phase. The result was

a significant lag in the formation of product (Figure 2-5) not previously observed in complex media.

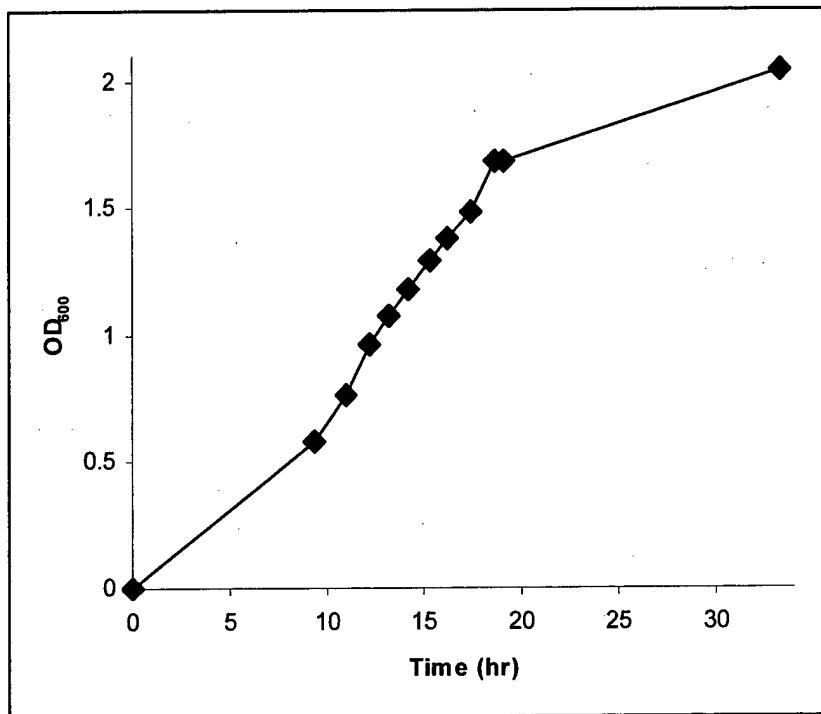


Figure 2-4. Standard growth curve for BL21(DE3)(pMM4) in M9 media. Biomass concentration is expressed as optical density of culture medium at 600 nm.

To combat the lag in lactone production, the culture was induced at the time of inoculation in M9 media followed by addition of substrate at an OD<sub>600</sub> of 0.6. The result was a biotransformation in which product formation was nearly linear (Figure 2-5) and cells displayed greater productivity per unit of biomass weight. This observation was explained by SDS-PAGE analysis of cells induced with IPTG and grown on M9 media (Figure 2-6). This revealed a slow appearance of CHMO over a five-hour period, which explains the lag in lactone production.

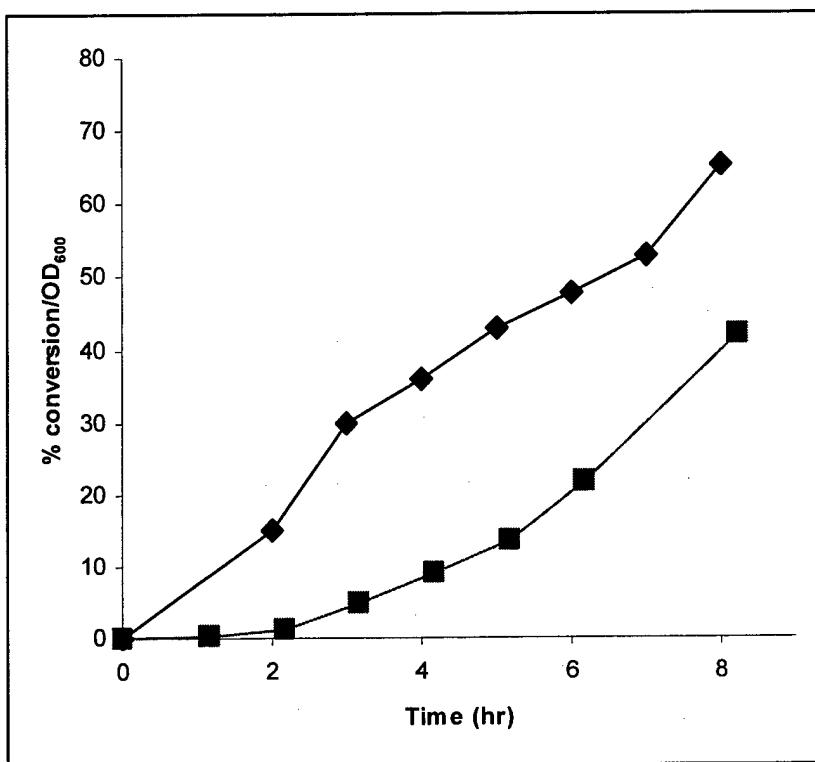


Figure 2-5. Product formation per unit of biomass. Cells grown in M9 and were induced at inoculation (◆) and cyclohexanone was added at 11 hours ( $OD_{600} = 0.6$ ). Cells grown in M9 and were induced at 10 hours ( $OD_{600} = 1.0$ ) (■) and cyclohexanone added at induction +20 min.

Unfortunately, biotransformations conducted in M9 medium still showed a total limit of approximately 10 mM lactone. Lactone production would also halt prior to 10 mM if the concentration of glucose in the media reached zero prior to substrate exhaustion.

Based on these preliminary studies, we identified two key issues: 1) the need to keep cells in log phase longer or prevent them from reaching stationary phase physiology, and 2) the need to maintain a supply of reducing equivalents (NADPH) via glucose feed.

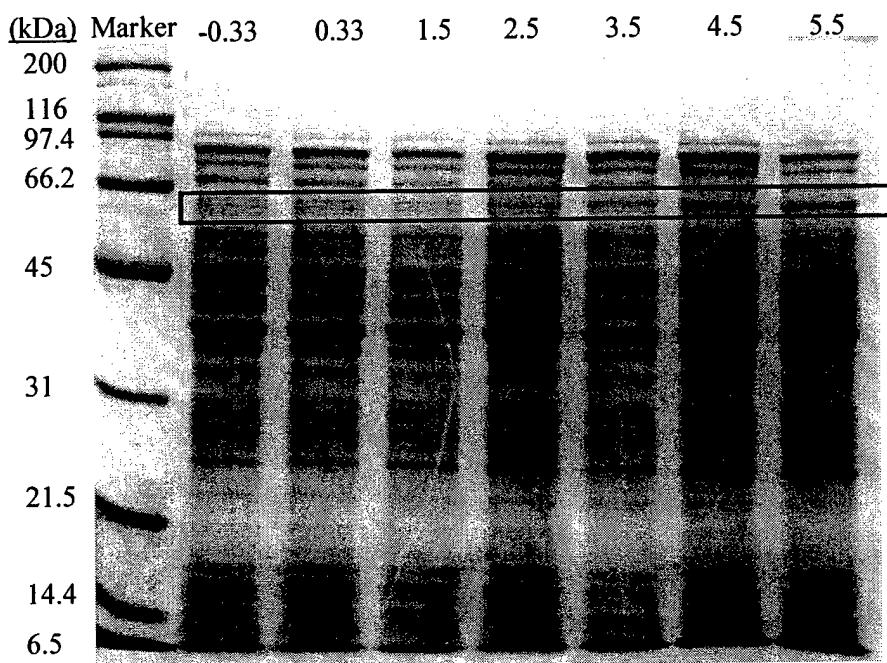


Figure 2-6. SDS-PAGE gel of IPTG induced cells grown in M9. CHMO band (~61 kDa) is indicated in lined box. Time post-induction is indicated above each lane.

### Biotransformation with Non-growing Cells

The concept for biotransformation with non-growing cells involves accumulating biomass to a high level (but not in stationary phase), then re-suspending the cells in a non-growth medium. The biotransformation would then be resumed in a fed batch scenario. The basic scheme for conducting non-growing cell biotransformations is outlined in Figure 2-7. Several factors were optimized prior to achieving desired results.

To prevent cell growth, NH<sub>4</sub>Cl was omitted from the reaction vessel, which contained otherwise complete M9 medium. Washed cells added to this media displayed no growth and a slow, linear loss of biomass (approximately 1% per hour) over the course of the reaction (Figure 2-8), presumably due to cell lysis.

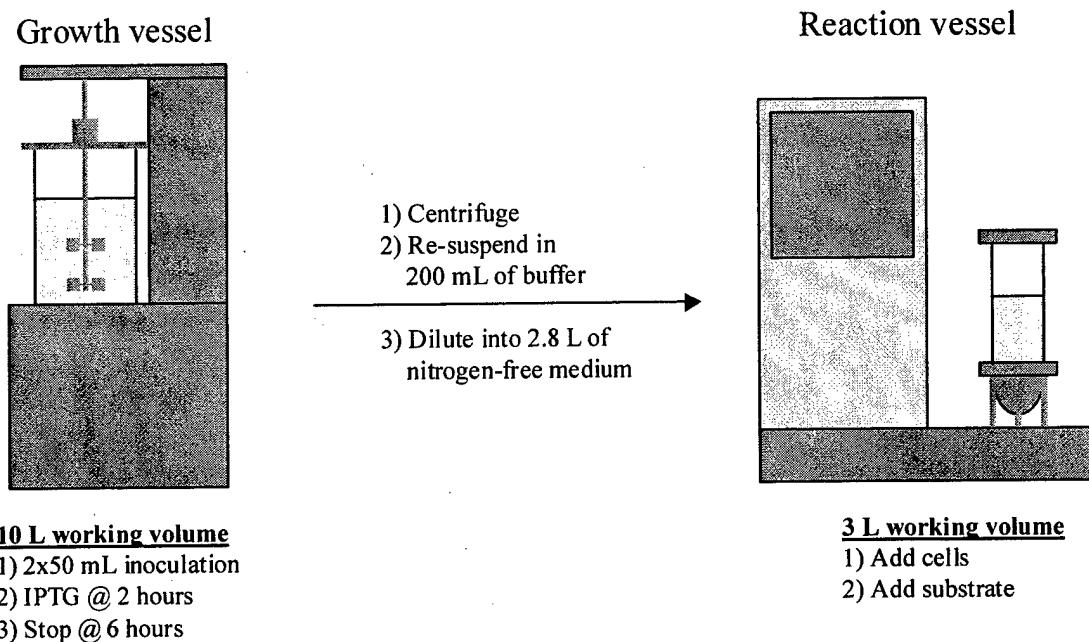


Figure 2-7. Schematic of non-growing cell method.

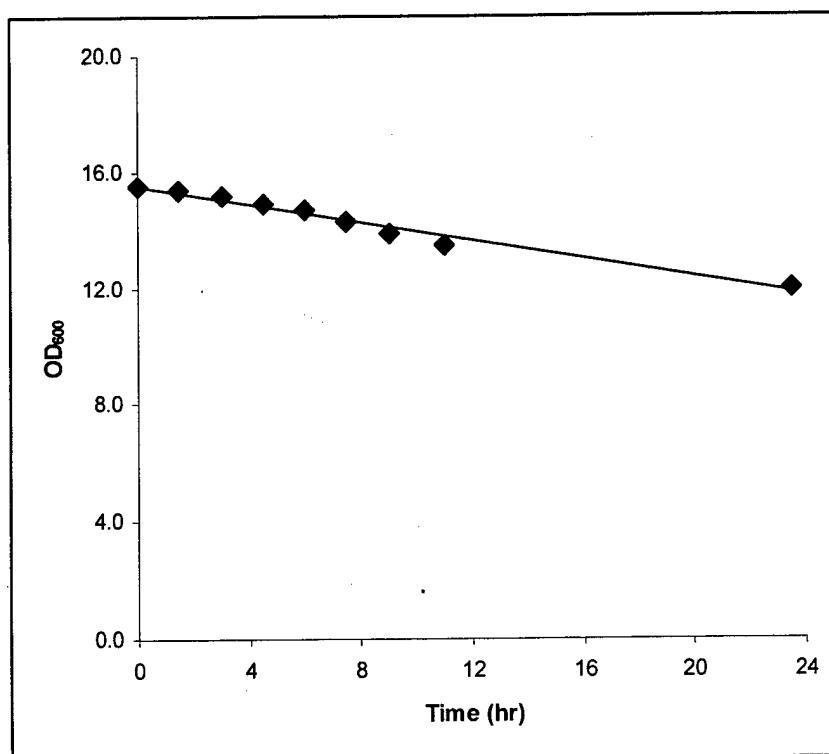


Figure 2-8. Loss of biomass during non-growing biocatalysis.

Next, the conditions under which the cells were prepared were optimized. Various combinations of LB media and M9 and its components were examined. Cells grown in each appeared to have nearly the same productivity per unit quantity of biomass when tested in 50 mL shake flask biotransformations in our non-growth medium. Ultimately, the medium that supported the fastest growth and highest biomass yield was selected for this purpose: LB supplemented with 0.4 g/L glucose (Figure 2-9).

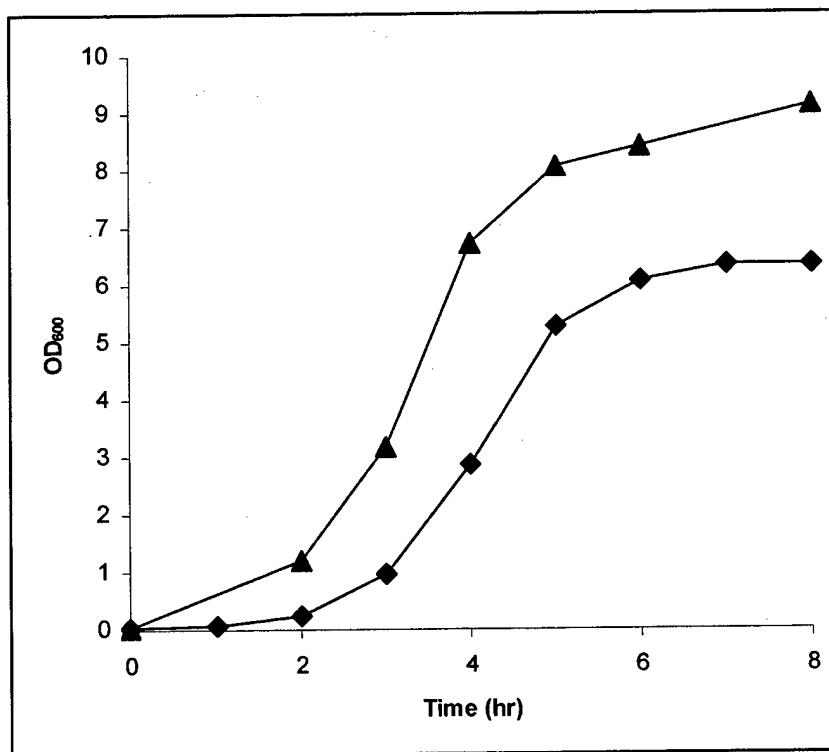


Figure 2-9. BL21 (DE3) growth in LB (◆) and LB + 0.4 g/L glucose (▲).

In the course of these investigations, we found that the time at which we harvested the cells was also critical for subsequent productivity under non-growing conditions. Cells harvested at various time points during biomass growth were washed and tested for productivity per unit biomass in small (50 mL) shake flask reactions (Figure 2-10).

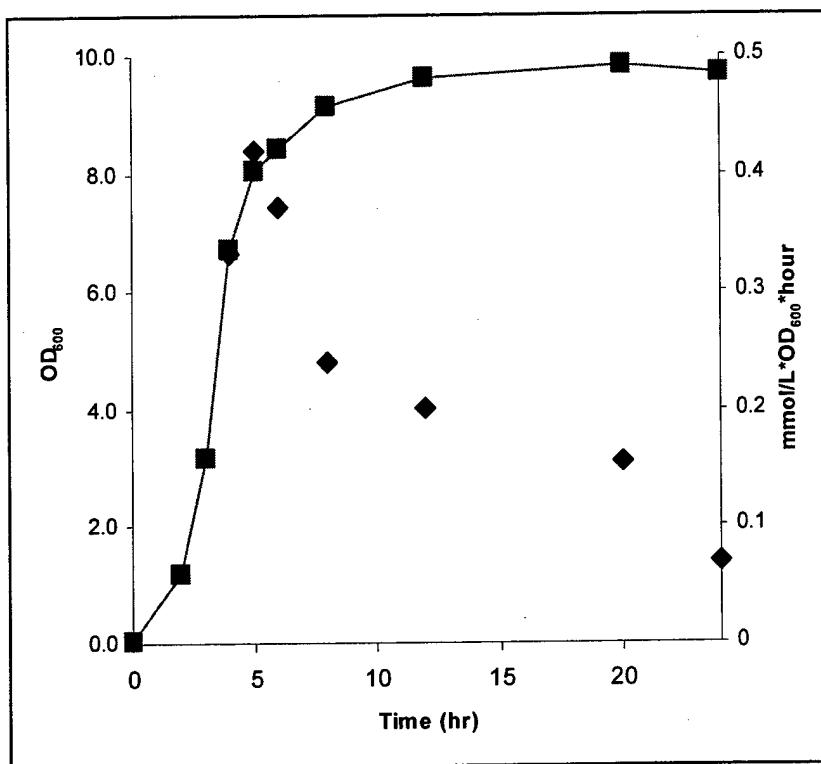


Figure 2-10. Biomass productivity over time under non-growing conditions. *E. coli* BL21(DE3) (pMM4) growth (■) was monitored over a 24 hour time period under optimized media conditions. Productivity (defined as mM lactone produced per unit biomass per hour; ◆) was determined via 50 ml shake flask non-growing cell biotransformations.

Subsequently, these observations were supported by cell crude extract enzyme assay data (Figure 2-11). These results led us to harvest cells at the transition between logarithmic growth and stationary phase, since these displayed the maximal enzyme activity and cellular productivity.

More careful investigation of the time period surrounding the growth phase transition showed no significant spike or drop in CHMO activity (Figure 2-12). The desired harvest time was generally at 6 hours post-inoculation or when cell growth dropped to less than one OD<sub>600</sub> unit change per hour.

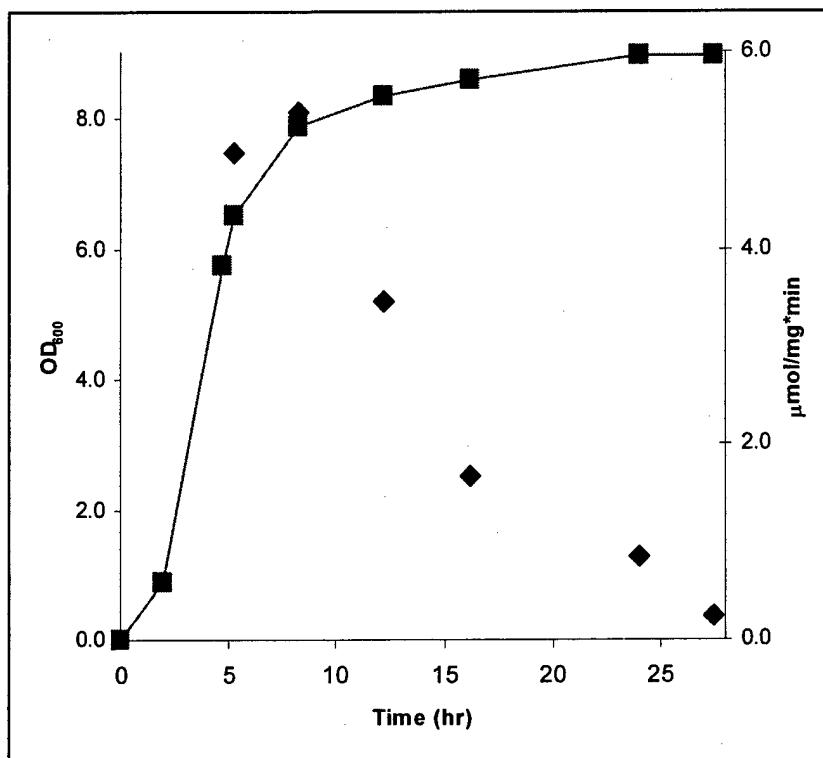


Figure 2-11. Enzyme activity over time under growing conditions. *E.coli* BL21(DE3) (pMM4) growth (■) was monitored over a 24 hour time period under optimized media conditions. Cellular crude extracts (◆) were analyzed for CHMO specific activity as a function of NADPH consumption measured by decrease in absorbance at 340 nm.

Finally, given that we were able to control the amount of cells introduced to the non-growing reaction medium, the optimal concentration of biomass needed to be determined. Biotransformations were conducted on a three-liter scale with a variety of starting biomass concentrations. It was determined that biomass loading much above an OD<sub>600</sub> of 12 yielded no significant improvement to the rate of lactone formation (Figure 2-13). Decreasing biomass below this level retarded the observed rates of reaction.

Non-growing cell biotransformations were then carried out under a variety of cyclohexanone feed strategies. The average maximum result for total lactone production over a 24-hour period was 70 mM, a 7-fold increase over our previously observed production limit (Figure 2-14).

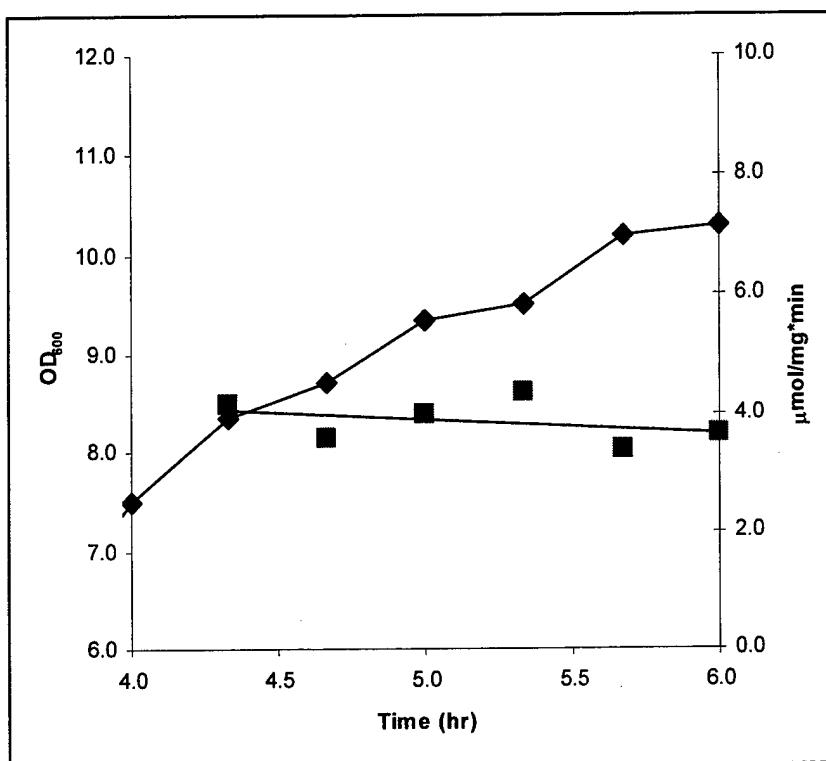


Figure 2-12. Enzyme activity during harvest time window under growing conditions. *E. coli* BL21(DE3) (pMM4) growth (◆) was monitored during the proposed harvest time window. Cellular crude extracts (■) were analyzed for CHMO specific activity as a function of NADPH consumption measured by decrease in absorbance at 340 nm.

Using this 70 mM benchmark as a reference, we made several attempts to improve on the productivity of non-growing cells. The first procedural change was to supplement the reaction medium with riboflavin in order to provide cells with a flavin source that could be assimilated to produce a necessary co-factor, FAD. The rationale behind this strategy will be discussed later in this chapter. Riboflavin-supplemented reactions yielded an average of 80 mM  $\epsilon$ -caprolactone, a 14% improvement over non-riboflavin-supplemented reactions (Figure 2-15). Note that the average values for non-supplemented media lie at the lower error limit of the supplemented data. However, average values for both lie within error of each other and it is thus unclear whether there

is truly a benefit to this modification. Under the assumption that riboflavin provided a general improvement, this inexpensive additive was adopted for subsequent experiments.

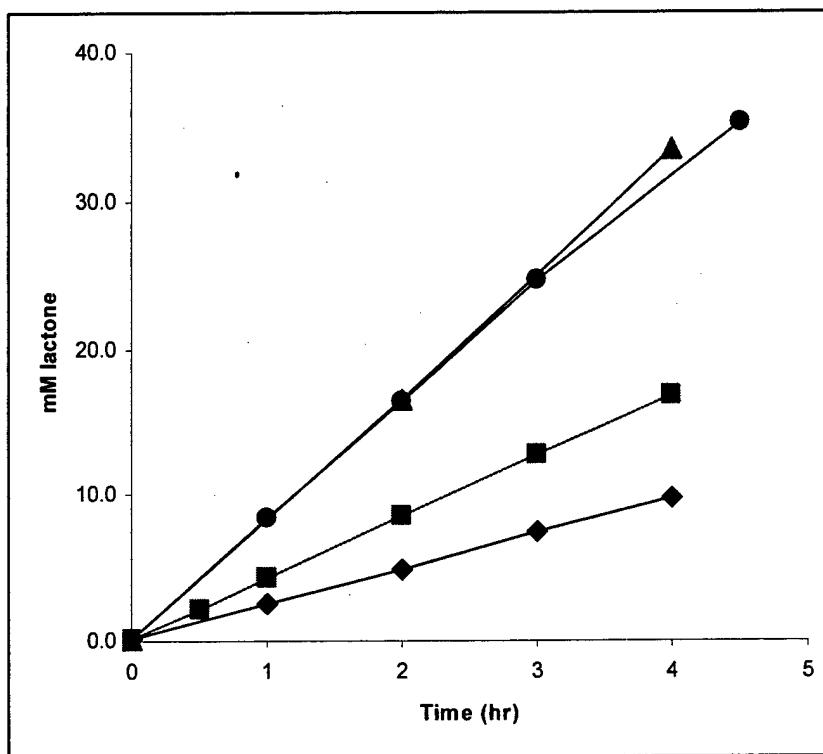


Figure 2-13. Cyclohexanone oxidation by non-growing cells at various initial cell densities. Four separate reactions are pictured at  $OD_{600} = 5$  (◆), 7 (■), 15 (▲), and 18 (●).

Given the observations noted in Figure 2-11, which indicated that enzyme activity appeared to degrade over time, we attempted to address this as a potential reason for observed productivity limits. Immobilized, non-growing cells have been shown to be effectively induced with IPTG in a nitrogen-free media (Swope and Flickinger, 1996). We hoped that transcription and translation of CHMO could occur at residual levels using scavenged intracellular amino acids. We therefore added IPTG to nitrogen-free minimal medium in an attempt to reverse, halt or slow enzyme degradation in our whole cells. Using this technique, values of up to 95 mM productivity were observed; however

average values indicated there was essentially no benefit from IPTG supplementation (Figure 2-16).

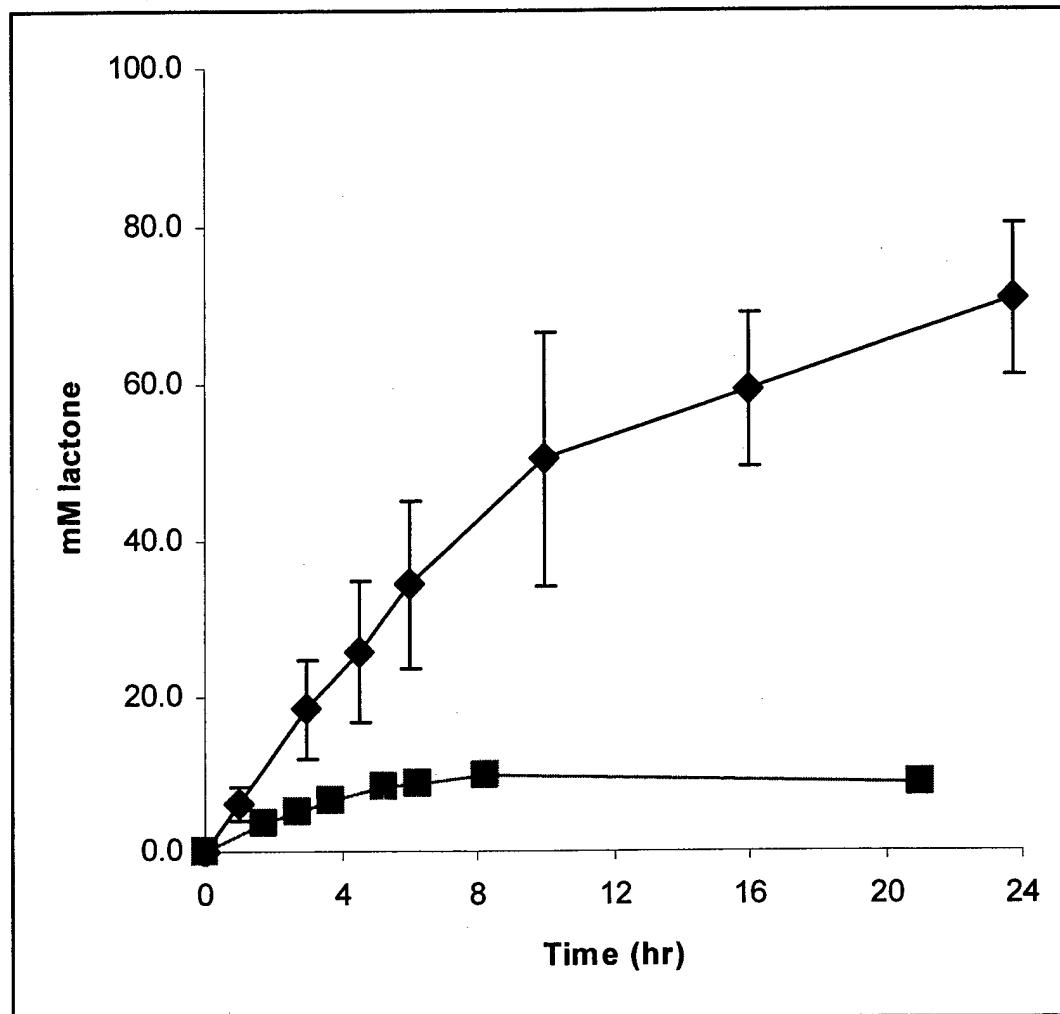


Figure 2-14. Non-growing cell productivity. Average non-growing cell productivity (◆) in M9 medium minus NH<sub>4</sub>Cl is compared to growing cell productivity (■) in LB medium with no glucose added.

Over the course of these experiments, it was noted that the productivity limit for an individual run was related to the initial rates of lactone formation in the first hour. In general, higher initial rates gave a higher total productivity (Figure 2-17). This lends credence to the theory that catalyst loss over time is a major contributor to observed productivity limits.

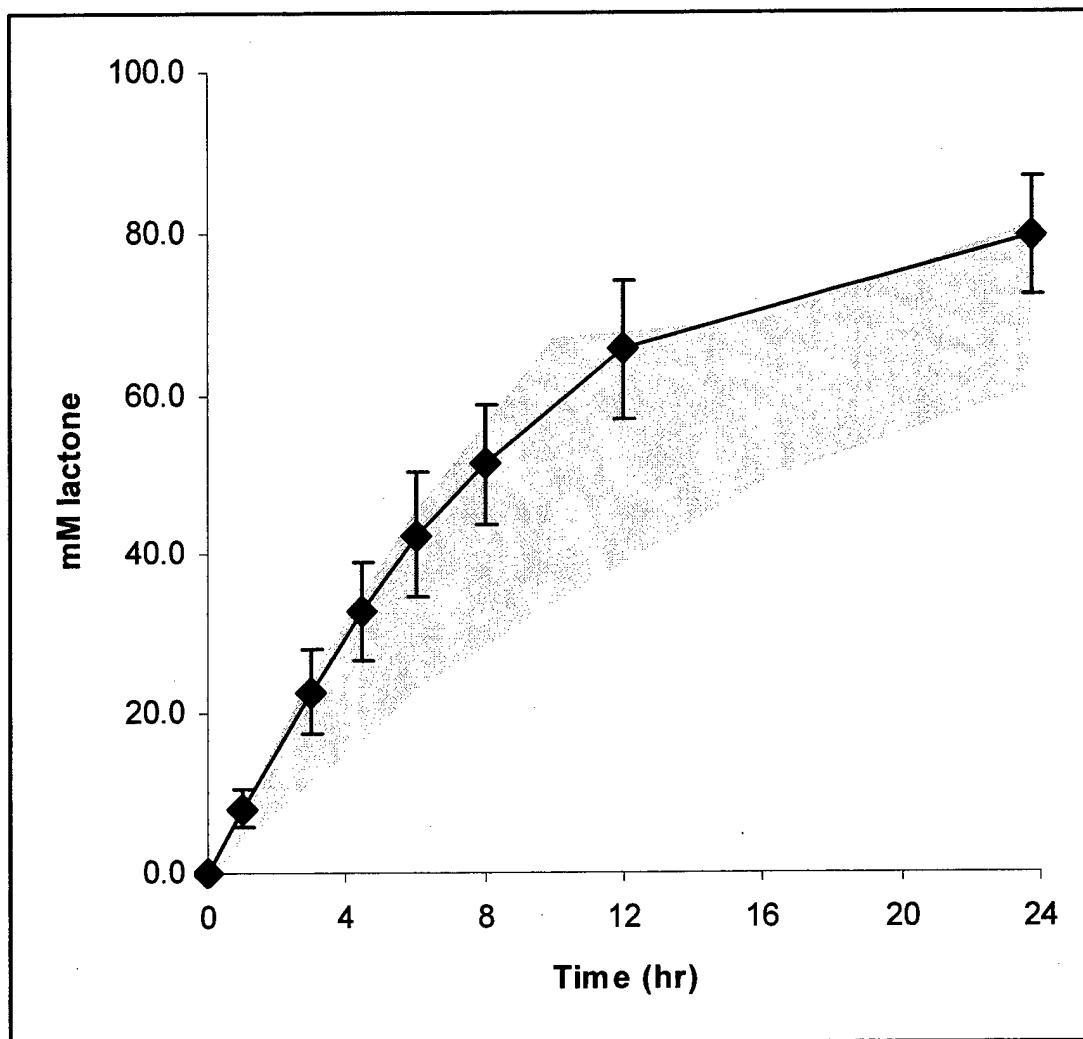


Figure 2-15. Riboflavin supplemented non-growing cell productivity. Average non-growing cell productivity (◆) supplemented with 1 mg/L riboflavin is compared to the range of standard deviation (shaded region) for reactions in non-supplemented media. Error bars represent the standard deviations from three replicate experiments.

It is our belief at this time that maximum yields for a non-growing cell biotransformation are limited to a value of around 80 mM. Attempts to proceed beyond this point will require, in part, addressing the issue of enzyme loss over time.

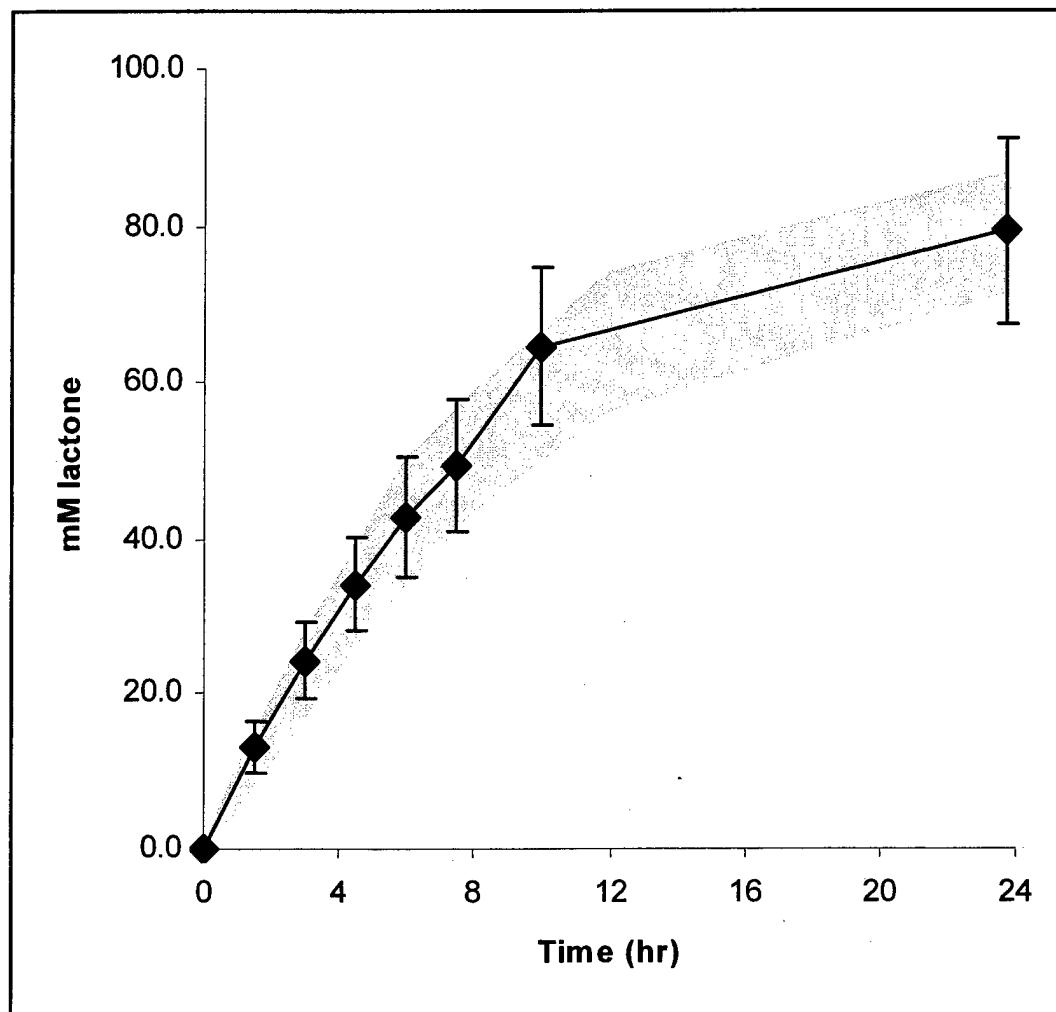


Figure 2-16. IPTG supplemented non-growing cell productivity. Average non-growing cell productivity (◆) supplemented with riboflavin and 0.1 mM IPTG is compared to the range of standard deviation (shaded region) for reactions in riboflavin supplemented media. Error bars represent the standard deviations from six replicate experiments.

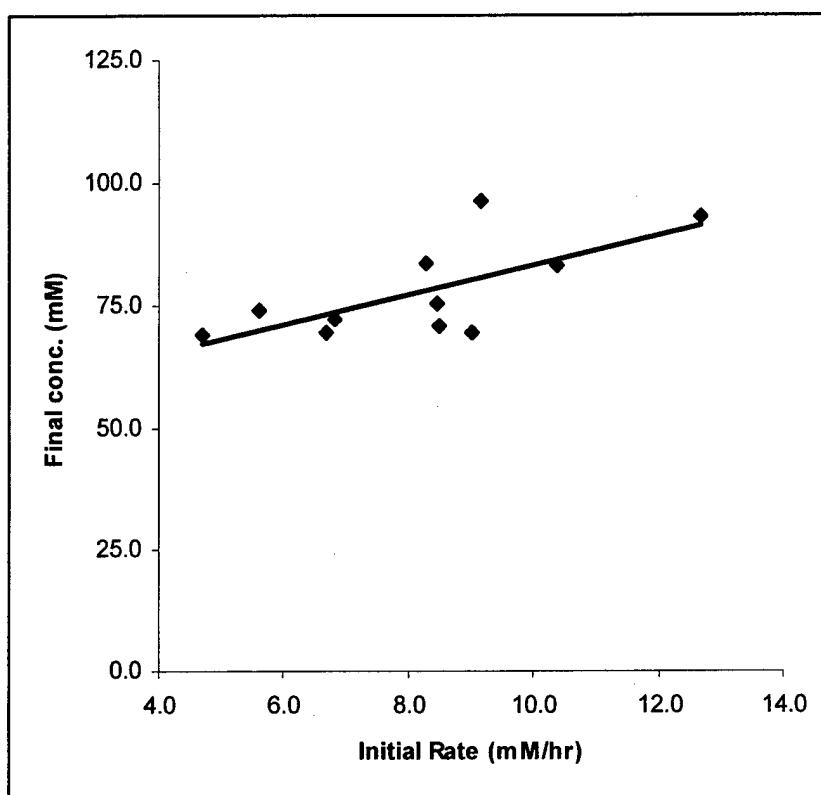


Figure 2-17. Total productivity as a function of initial rate.

#### Biotransformation in LB with Glucose Supplement

An additional factor, not originally considered, was the addition of glucose as a source of NADPH reducing equivalents to our original complex media. Maintenance of a pseudo-steady state of glucose (0.4 g/L or ~22 mM) in LB medium was therefore investigated as a way to accomplish cell growth and cyclohexanone oxidation in a single unit operation. When glucose addition was combined with slow feed of cyclohexanone (via pump) the result has been an average 90 mM lactone (Figure 2-18). This value is also comparable to the maximal values found with non-growing cells. We believe this to be the best value currently obtainable for any fed-batch CHMO bioconversion. The ability to perform this reaction in a single vessel, without time and materials involved in a separate biomass production, while still achieving the same or better yield, make this

method preferred over the non growing cells technique. This productivity limit, which appears linked to a specific concentration of  $\epsilon$ -caprolactone, may result from product inhibition. This, along with enzyme stability, will be investigated in the next section.

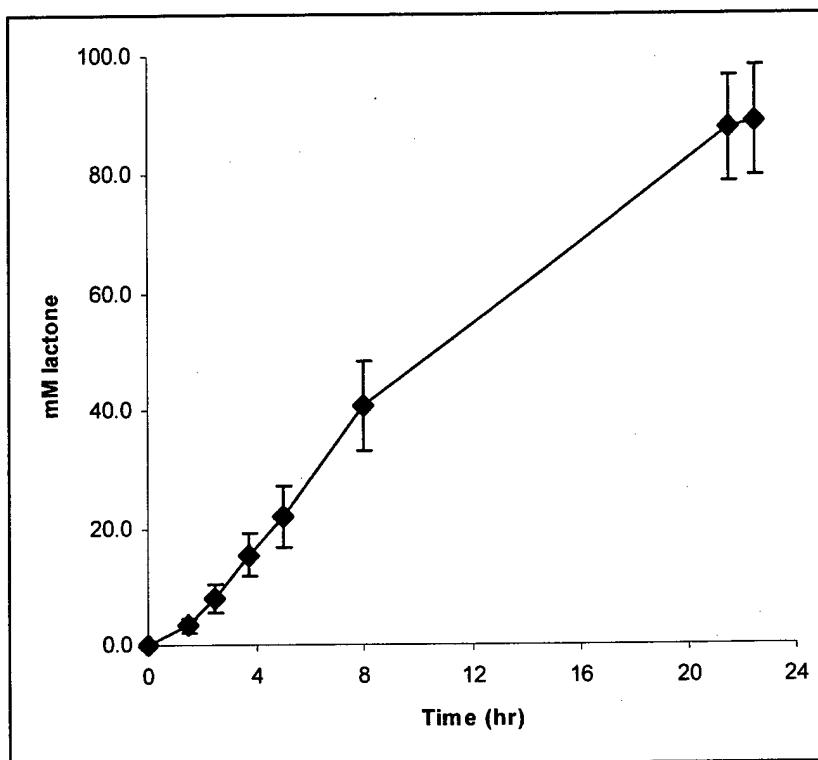


Figure 2-18. LB + glucose growing cell productivity. Data is shown as an average of three separate biotransformations.

### Factors Affecting Whole Cell Performance

The work described in the previous section suggested that several aspects of the bioconversion should be examined as potential explanations for whole cell productivity limits. This section will cover what we know about these issues that include enzyme stability, substrate and product concentrations, oxygen and co-factors.

#### CHMO Enzyme Stability

Cyclohexanone monooxygenase loss over time is the most likely explanation for the limited productivity of the engineered whole cells. As a part of the monitoring

process, CHMO activity in crude extracts was determined by following NADPH oxidation in the presence of cyclohexanone (Figure 2-19). The data depicted in Figure 2-19 display significant variation among runs of the same type. However, at a minimum, it can be seen that CHMO experiences a loss of at least 90% of its activity over a 24 hour period when exposed to reaction conditions. This decrease is independent of the specific reaction media and the presence or absence of cyclohexanone. Furthermore, this phenomenon appears to be due to actual enzyme loss, as opposed to enzyme inactivation, since visual analysis of SDS-PAGE gels consistently showed a parallel decrease in intensity of a band at the MW corresponding to CHMO (Figure 2-20).

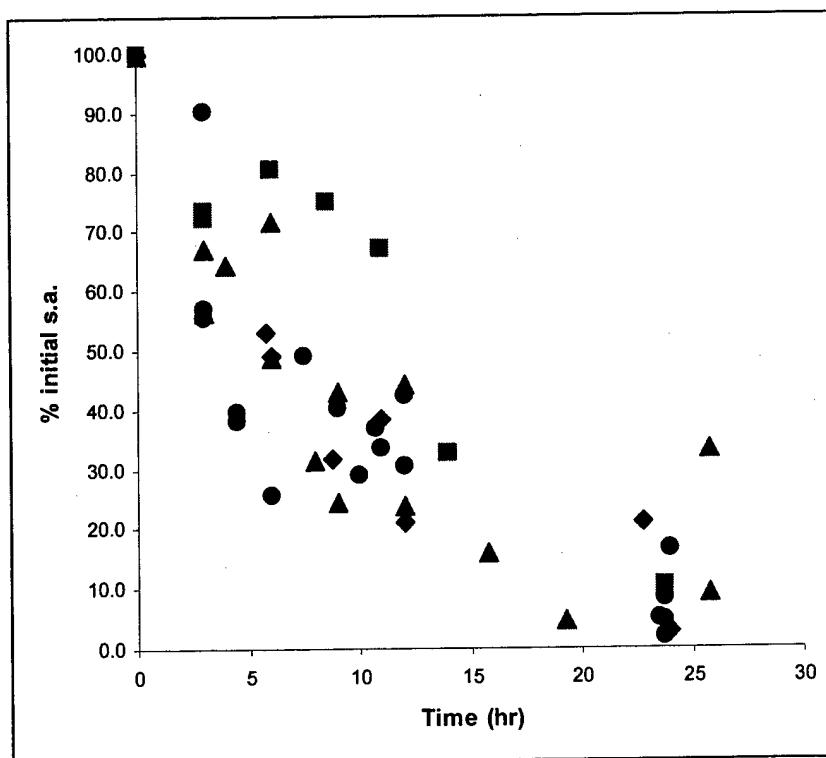


Figure 2-19. CHMO specific activity in crude extracts during bioconversions under non-growing conditions. Specific activity measured by NADPH oxidation displayed as a percent of each experiment's time zero value. Non-supplemented (♦), riboflavin supplemented (■), riboflavin + IPTG supplemented (●) and cyclohexanone omitted (▲) reactions are depicted.

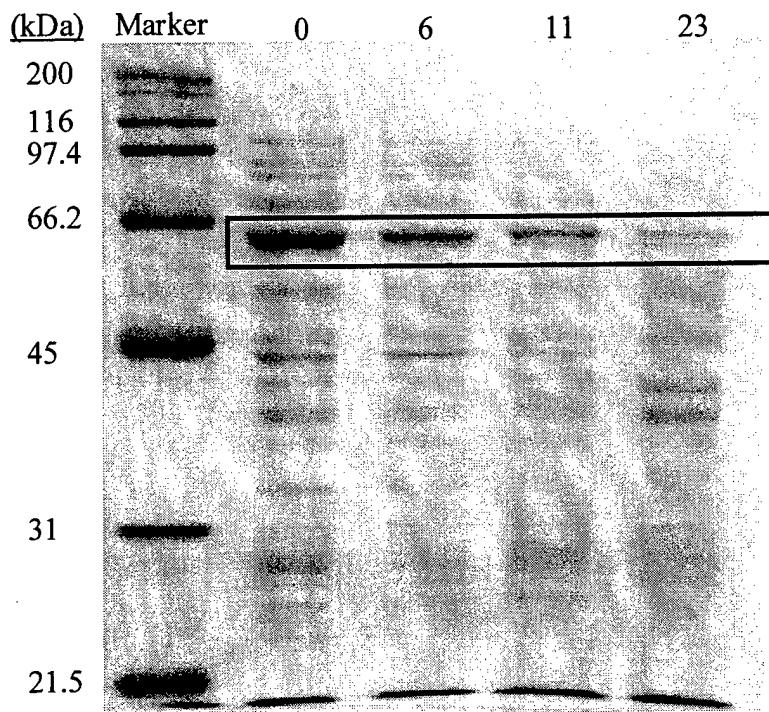


Figure 2-20. SDS-PAGE gel of typical enzyme loss profile. Hours post-substrate addition are indicated above each lane.

Recently published work has demonstrated that minimizing dissolved oxygen tension and maximizing oxygen uptake rates during biomass growth has a significant impact on yields of CHMO enzyme in a related *E. coli* strain (Doig *et al.*, 2001). This work, which focused on optimizing enzyme production for subsequent purification, proposed that excess oxygen facilitates inactivation of the enzyme by oxidizing an active site –SH group (Figure 2-21). Early enzyme studies (Donoghue and Trudgill, 1976) suggested that CHMO contains a catalytically important sulfhydryl group near the reaction center, but its role (if any) in the mechanism is unknown. Analysis of other known Baeyer-Villiger mono-oxygenase sequences listed in GenBank revealed no conserved cysteine residues across the enzyme family (data not shown). Sulfhydryl modification and subsequent enzyme inactivation followed by proteolysis could explain

the loss of enzyme over time. Moreover, if this mechanism of inactivation is valid, other BVMO enzymes may display different degradation profiles and therefore lead to potentially higher productivity yields.

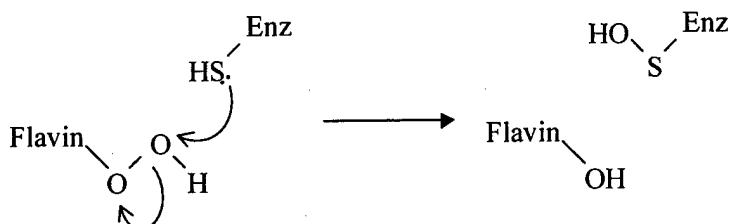


Figure 2-21. Proposed mechanism of -SH oxidation.

Proteolysis under our reaction conditions is another possibility for the observed loss of CHMO. It has also been observed that when *E. coli* enter into nutrient starvation – whether because of entering stationary growth phase or by removal of a key nutrient – there is a five-fold increase in protein turnover (Kolter *et al.*, 1993). During this turnover, the cells break down proteins previously needed for rapid growth and synthesize those that enable long-term survival. It is possible that our recombinant protein is a target of this survival mechanism, whether because of prior sulphydryl modification or because it is not essential for cell survival.

To test the theory that proteins expressed during starvation phase might impact the observed lifetime of CHMO we conducted an experiment in which chloramphenicol was added to the non-growing cell reaction medium at the time biomass was introduced. The introduction of 170 mg/L chloramphenicol was designed to inhibit translation and should stop all new protein expression during the bioconversion under non-growing conditions. The result was a biotransformation where normal cyclohexanone oxidation occurred up until approximately 11 hours, at which time we deduce that the cells became metabolically inactive due to a sudden halt in glucose consumption. The enzyme loss

profile, however, appeared unaffected by the inhibition of protein expression (Figure 2-22). This indicates that any new proteins expressed during this artificially induced starvation have little impact on the loss of CHMO over time.

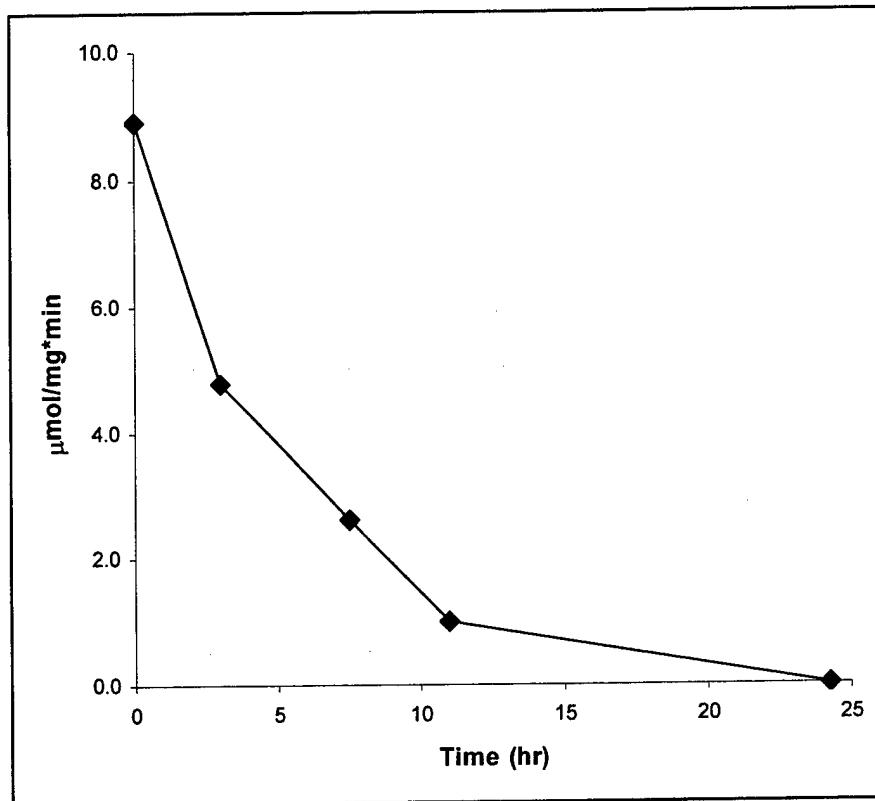


Figure 2-22. Enzyme activity of CHMO under non-growing conditions in the presence of chloramphenicol.

To rule out general proteolysis by the BL21(DE3) strain as the major reason for CHMO activity loss, data for this Baeyer-Villiger monooxygenase was compared to those of an unrelated NADPH-dependent enzyme expressed in the same strain (Figure 2-23). BL21(DE3)(pAA3) overexpresses the NADPH dependent yeast reductase Gre2 (Rodriguez *et al.*, 2001) and displayed a much more gradual enzyme loss profile. This demonstrates the dramatic variation in loss behavior that is specific to individual enzymes.

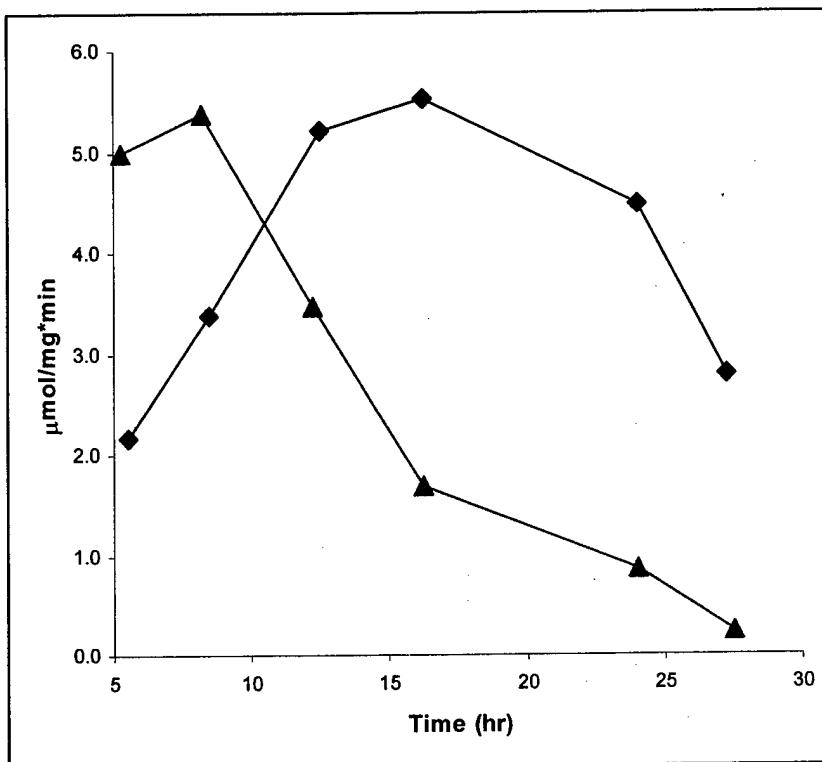


Figure 2-23. Enzyme activity of CHMO (▲) and Gre2 (◆). Both enzymes are over-expressed in the same host (BL21(DE3)) which displays the same growth behavior regardless of expression plasmid. This data is taken from crude extract analysis of cells grown in LB + 4 g/L glucose allowed to progress into stationary phase growth.

#### Product and Substrate Effects

Product inhibition was investigated as a factor that might have been responsible for the observed loss of CHMO activity. The presence of the cell membrane as a fundamental interference in traditional kinetic experiments as well as the desire to test the same batch of cells under exactly the same reaction conditions made this experimental design a challenge. We chose to introduce 15 mM  $\epsilon$ -caprolactone aliquots to the fermentation vessel at one-hour intervals up to 5 hours and measured the observed hourly rate change between these additions up to a period of 8 hours. When compared to the hourly rate loss of cells not subjected to artificial lactone additions, we observed a 25% faster decrease in rate over time as well as approximately 25% less lactone produced over

an 8-hour period (Figure 2-24). While no inhibition constant for  $\epsilon$ -caprolactone and CHMO has been reported to date (Sheng *et al.*, 2001) it is possible that the complex nature of the whole cell environment and the relatively high concentrations of lactone used here may provide a real effect on the productivity limit of the whole cell method. Although the effect observed in Figure 2-24 is slight, observations that an upper limit of 90 mM truly exist support this concentration dependence theory.

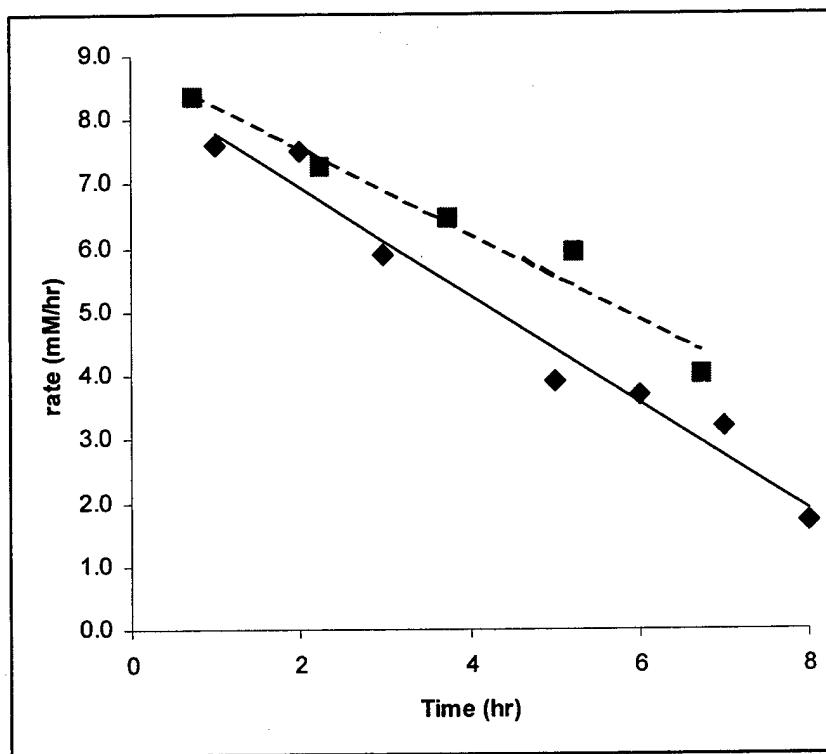


Figure 2-24. Effect of lactone concentration on rate loss. Average hourly rates of cyclohexanone oxidation in  $\epsilon$ -caprolactone supplemented biotransformation (◆, solid line) and non- $\epsilon$ -caprolactone-supplemented biotransformation (■, dashed line). Both biotransformations are run under three-liter non-growing conditions. Lactone supplementation was conducted by adding product in 15 mM aliquots at each hour from 1-5 hours.

The effect of cyclohexanone concentration on the reaction rate was not formally investigated. The experience in our group has been that growing cells respond poorly to cyclohexanone concentrations greater than 10 mM. In this project, it was observed that

substrate concentrations of 30 mM have neither a positive or negative effect on rate or productivity of the same cells under non-growing conditions. From this, 30 mM was chosen as an upper limit for bolus and steady feed cyclohexanone additions with non-growing cells. A value of 15 mM was chosen arbitrarily for the limit in glucose supplemented growing cell reactions.

At 10 mM, cyclohexanone levels are much higher than the reported  $K_m$  (4  $\mu$ M) for CHMO (Donoghue and Trudgill, 1976). This can be interpreted to mean that intracellular substrate concentrations would have to lie below 4  $\mu$ M in order to be rate limiting. Since this seems unlikely, it was not investigated in this work.

Limiting the cyclohexanone concentration also reduced the amount of the volatile substrate lost to the environment under the high air-flow rates used. Environmental loss of up to 25% of cyclohexanone added has been observed. This further supports the selection of such an inexpensive substrate for this type of study.  $\epsilon$ -Caprolactone displays much less sensitivity to aeration conditions and its loss due to stripping was negligible under our experimental conditions.

### **Oxygen**

Oxygen, which was suspected to have a detrimental effect on enzyme stability, has also been shown to be critical in achieving maximal productivities (Doig *et al.*, 2001). Oxygen is a substrate in the Baeyer-Villiger reaction, and if dissolved oxygen was allowed to reach zero in the bio-reactor, significant loss in reaction productivity was observed. This phenomenon was observed early in the course of this work, which led to the choice of 25% saturation as the maximal dissolved oxygen set point. This level served two purposes. First, it allowed for sudden downward fluctuations to be managed

without accidentally reaching the zero point. Secondly, it allowed for generally lower airflow than at higher set points and therefore reduced the amount of cyclohexanone lost by stripping.

Lowering the dissolved oxygen set-point to 5% saturation in the bio-reactor had no negative effect on reaction rates. Donoghue estimated the  $K_m$  for oxygen as  $<100 \mu M$  (Donoghue and Trudgill, 1976). Of course, CHMO is not the only intracellular enzyme requiring oxygen, and these may out-compete and therefore limit CHMO rates in whole cells. Intracellular oxygen concentrations were not investigated in this research.

### **NADPH**

An efficient source of NADPH is critical for extended biotransformations with cyclohexanone monooxygenase. Glucose addition was the single change that allowed for the nearly 10-fold improvement in lactone yield with growing cells in LB medium. Most likely, this is due to glucose's ability to feed the NADPH pool through the pentose phosphate pathway. It is conceivable that LB media, by the time cells reach the stationary phase, may be depleted in its ability to feed the NADPH demand of CHMO.

We do not believe that the reaction rate of CHMO under our conditions is limited by the rate of NADPH supply. This notion is supported by our observation that ethyl acetoacetate reduction to the corresponding  $\beta$ -hydroxy ester by Gre2 expressed in *E. coli* consumes NADPH at the same ratio as the CHMO mediated oxidation of cyclohexanone, yet ketone reduction proceeded at nearly twice the rate found for cyclohexanone oxidation by non-growing cells under essentially identical conditions (Figure 2-25). This indicates that the BL21(DE3) cells are capable of generating NADPH at a faster rate when this is required. This example also demonstrated the fact

that our non-growing cell method can also be easily applied to other NADPH dependent reactions.

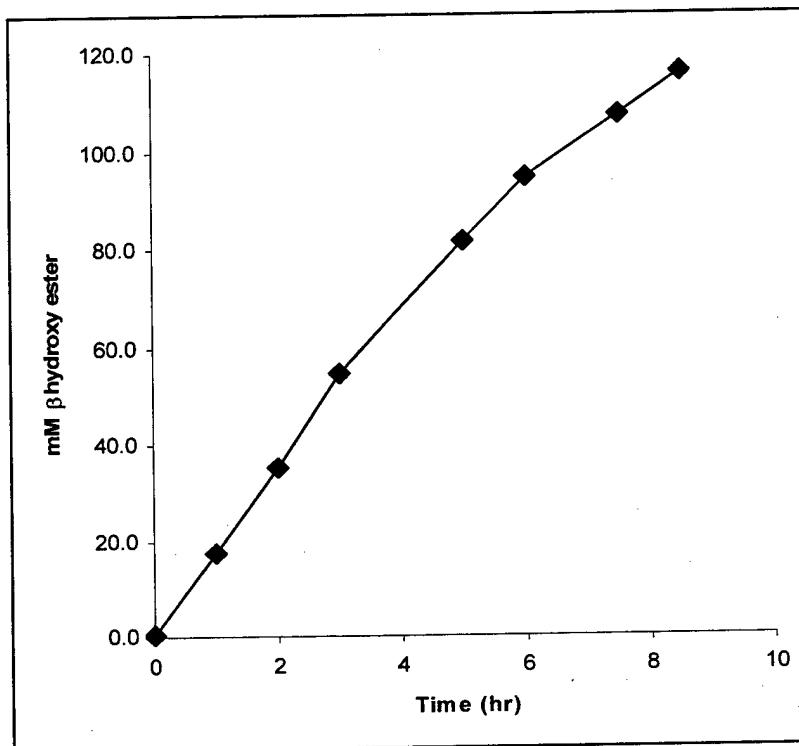


Figure 2-25. Product formation of Gre2. Biotransformation was conducted using the non-growing cell technique with the strain BL21(DE3)(pAA2).

We also examined the levels of nicotinamide co-factors over the course of a non-growing cell biotransformation to address the possibility of their depletion, which would prevent the desired Baeyer-Villiger oxidation. Cycling assays for determination of nicotinamides were based on the method published by Bernofsky and Swan in 1973 (Figure 2-26). Actual protocols used were identical to the methods developed by Gibon and Larher in 1997 for determination of nicotinamides in plant tissues. This method yielded values consistent with those published in the literature (Lilius *et al.*, 1979). The assay technique used allowed for quantitation of  $\text{NAD}^+$  and  $\text{NADP}^+$  in both oxidized and reduced forms.

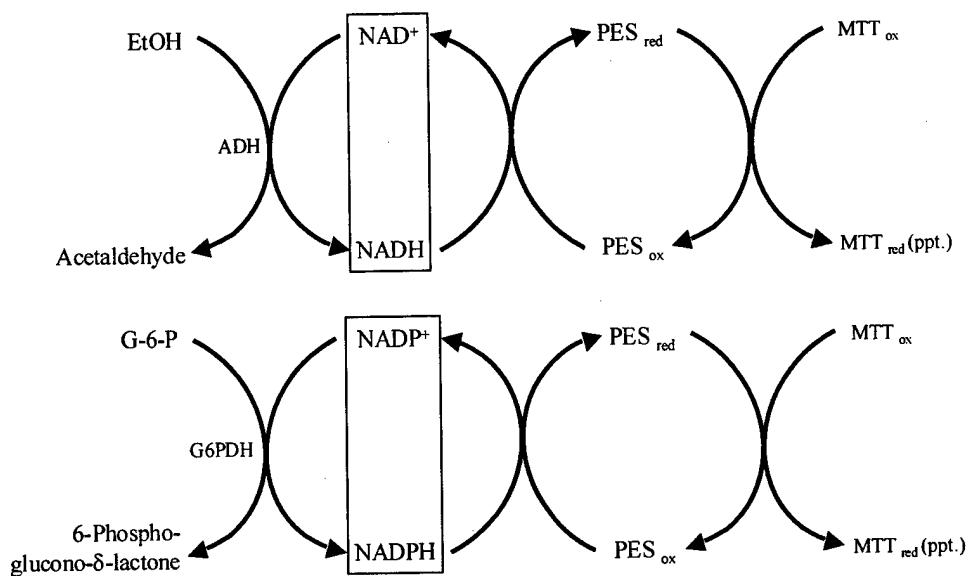


Figure 2-26. Nicotinamide cycling assay scheme. The quantity of reduced MTT precipitate formed over time is directly proportional to the concentration of co-factor present regardless of oxidation state.

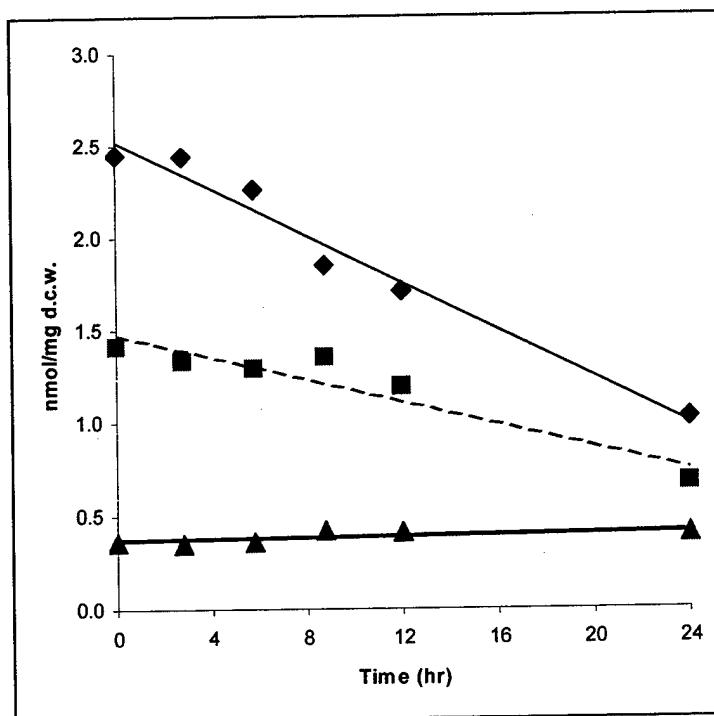


Figure 2-27a. NAD(H) assay during non-growing cell biotransformation. Oxidized (◆, solid line), reduced (■, dashed line), and fraction of the total in the reduced form (▲, heavy line) are depicted over a typical 24-hour reaction.

Our data showed that  $\text{NAD}^+$  and  $\text{NADP}^+$  concentrations dropped slowly and linearly over a 24-hour reaction (Figures 2-27 a and b). During this period, however, the fraction of each in the reduced form as well as their ratio to one another remained nearly constant. This demonstrates that the host organism seeks to maintain a balanced pool of co-factor over time and that loss of NADPH is not a significant reason that cyclohexanone oxidations ceased after *ca.* 20 hours.

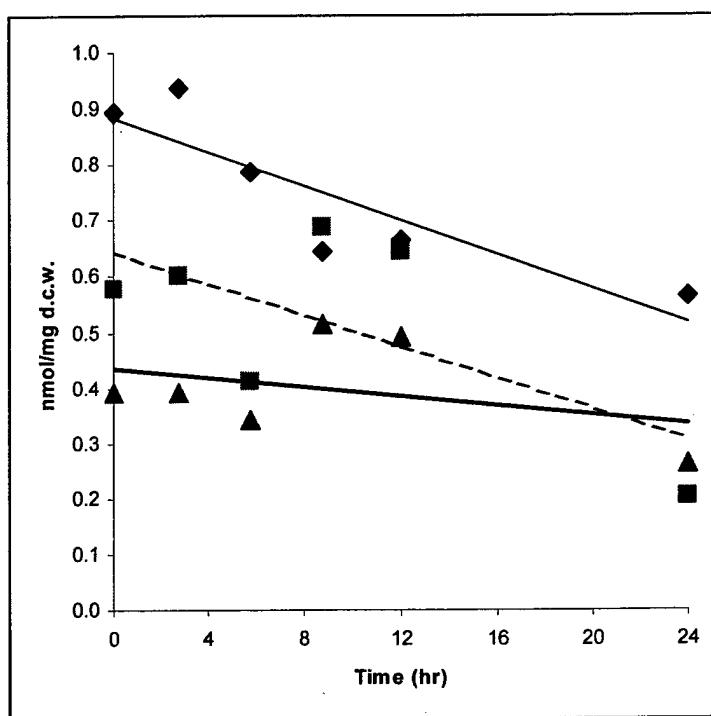


Figure 2-27b. NADP(H) assay during non-growing cell biotransformation. Oxidized (◆, solid line), reduced (■, dashed line), and fraction of the total in the reduced form (▲, heavy line) are depicted over a typical 24-hour reaction.

These data also allowed us to determine the intracellular concentration of NADPH (Figure 2-28). Using the average NADPH concentration and biomass density over the first twelve hours, we determined the approximate NADPH concentration to be 177  $\mu\text{M}$ :

$$\frac{0.58 \mu\text{mol NADPH}}{\text{g d.c.w.}} \times \frac{2.9 \times 10^{-13} \text{ g d.c.w.}}{\text{cell}} \times \frac{1 \text{ cell}}{9.5 \times 10^{-16} \text{ L}} = \frac{177 \mu\text{mol NADPH}}{\text{L}}$$

Figure 2-28. Calculation of intracellular NADPH concentration. Assumes the average dry weight per cell (Neidhardt, 1990) and the estimated intracellular volume of a rod-shaped *E. coli* cell (Nelson and Cox, 2000).

The reported *K<sub>m</sub>* of CHMO for NADPH is 20  $\mu\text{M}$ . Assuming that the majority of the NADPH pool is accessible to the enzyme, it appears that the cell provides more than sufficient levels of NADPH for the biotransformation.

#### Flavin Adenine Dinucleotide (FAD)

The flavin effect on Baeyer-Villiger oxidation was investigated indirectly by the addition of riboflavin to the non-growing cell reactions. This was prompted by discovery that adding FAD to crude enzyme assays had the consistent effect of doubling the observed specific activities (Figure 2-29). This effect may be an artifact of cell breakage and it is likely that adequate concentrations of intracellular FAD are present in the cytoplasm to saturate CHMO since the *K<sub>D</sub>* is known to be *ca.* 40 nM (Donoghue and Trudgill, 1976). Nonetheless, by providing a flavin source for the cells via the inexpensive additive riboflavin, we appear to have slightly enhanced the non-growing cell system. All crude extract assays included added FAD.

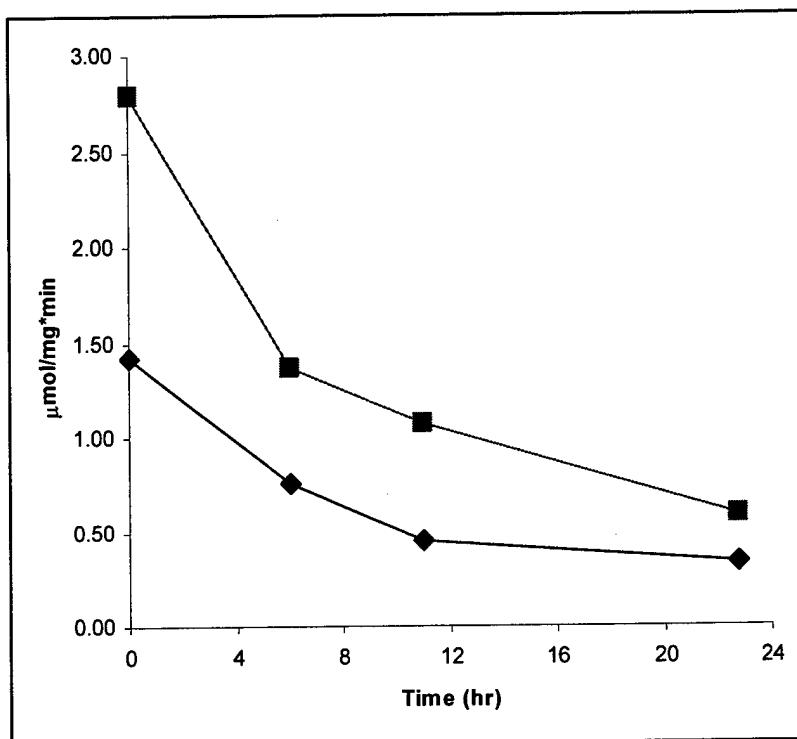


Figure 2-29. Typical FAD assay effect in CHMO activity crude extracts. Assays run without FAD addition (◆) are shown to approximately double in value (■) when supplemented with 15  $\mu\text{M}$  FAD.

### Product Recovery

The technique used for product isolation from these relatively large three-liter reactions was derived from a method originally intended to remove the  $\epsilon$ -caprolactone from the reaction vessel and thereby reduce product inhibition. Several examples have been published where resins have been used as a “second phase” in a bi-phasic reaction in order to limit toxic effects of substrate and product (Vicenzi *et al.*, 1997). Most of these employ the use of resins in the reaction vessel to perform this task. This technique is not appropriate in our case because the rapid stirring, required to maintain proper oxygen levels, would lead to cell lysis by shearing (Zmijewski, M.J., personal communication).

To overcome this problem, we developed a scheme in which the entire reaction mixture could be exposed to a non-polar support under more gentle conditions. In our method, the material was pumped through an inverted flow column containing a hydrophobic resin and then re-circulated back to the reaction vessel (Figure 2-30). Several solid support resins were investigated for this purpose. We found that Amberlite XAD 4 resin had the greatest capacity for adsorption of  $\epsilon$ -caprolactone and it was therefore selected as our resin of choice.

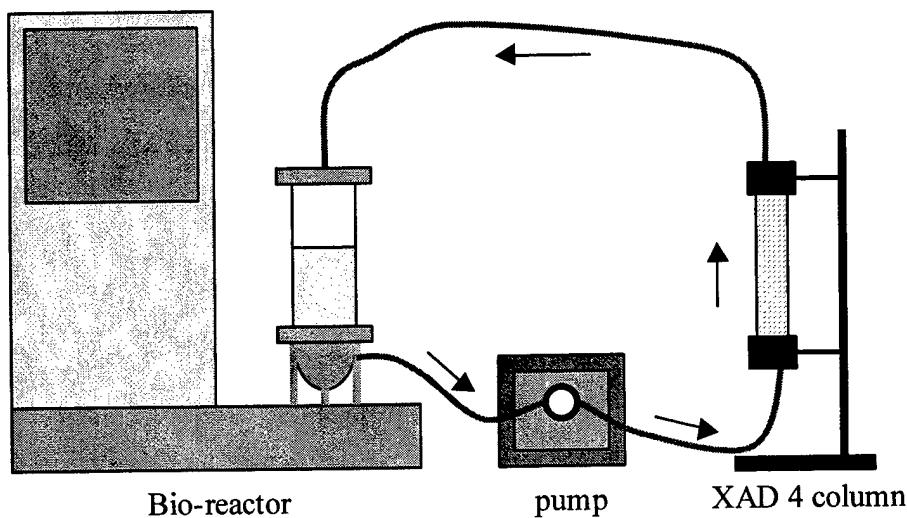


Figure 2-30. Schematic diagram of product recovery method.

Using the methodology outlined in Figure 2-27, a full term biotransformation was re-circulated four times over 200 grams of XAD 4 resin for 30 minutes. Between each re-circulation, the resin was washed once with solvent and once with clean water. Four re-circulations provided a 90% reduction in product concentration in the reaction vessel (Figure 2-31). Product was then extracted and distilled from these wash cycles for a 74% final, isolated yield of  $\epsilon$ -caprolactone.

Cyclohexanone is less polar than our lactone product and would have been adsorbed preferentially, thereby lowering the resin's capacity for  $\epsilon$ -caprolactone. For this reason, it was critical that all the substrate was exhausted prior to removing product by this method.

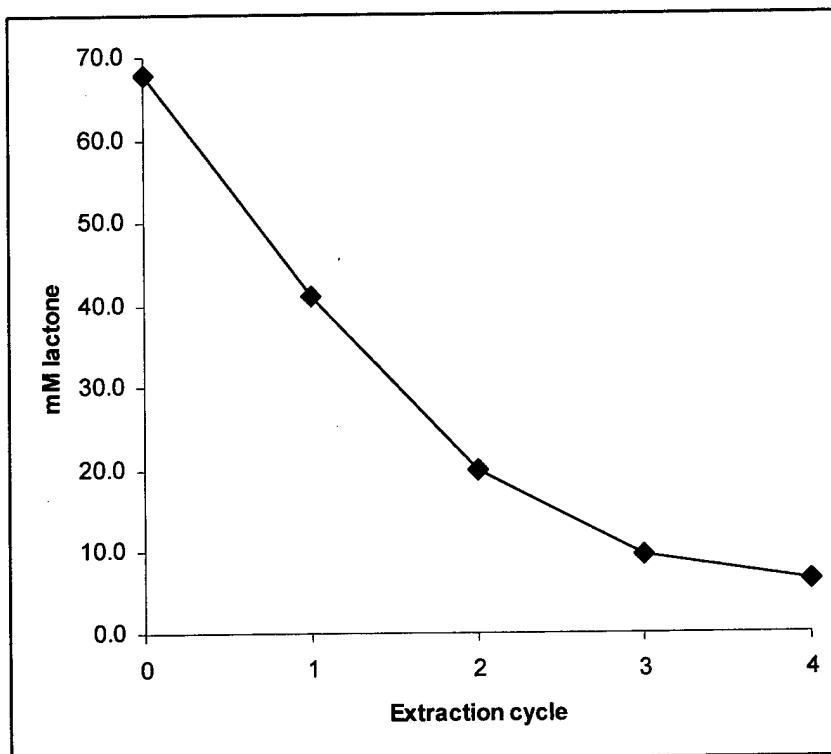


Figure 2-31. Lactone recovery by resin adsorption. Concentration of product remaining in the reaction vessel is plotted as a function of number of 30-minute re-circulation cycles. Four cycles captures 90% of  $\epsilon$ -caprolactone present at the conclusion of this whole non-growing cell reaction.

Once this method of product removal had been established, it was used to test the possibility that product inhibition was a significant cause of the decrease in bioconversion observed after *ca.* 14 hours. In this attempt, 30 mM bolus additions of cyclohexanone were administered to non-growing cells. After all of the substrate had been consumed, the mixture was pumped over XAD-4 resin to remove approximately 50% of the lactone product after each passage. A fresh 30 mM ketone bolus was then added and the process

was repeated. Unfortunately, this approach failed to yield greater productivity. Instead, the time lost for product recovery during our catalytic “window of opportunity” resulted in slightly lower overall  $\epsilon$ -caprolactone yield.

### Conclusion

In conclusion, we have demonstrated methods for improving the overall volumetric productivity of the biocatalytic oxidation by nearly 10-fold. We made these improvements by introducing basic environmental controls and the inclusion of inexpensive additives. While the initial cost of the bio-reactor is not insignificant, the added glucose that promoted this vast improvement in productivity added less than 25 cents per liter to the process cost at our laboratory scale. Dramatic cost reduction per mole of product is also seen when our method is compared to those requiring purified enzymes and co-factor regeneration methods. In short, whole cell catalysis is now better poised to move to the forefront of enzymatic synthesis.

Additionally, we have demonstrated that this biotransformation can be conducted to nearly the same efficiency using non-growing cells at a rate of *ca.* 20  $\mu\text{mol}/\text{min}\cdot\text{g}$  d.c.w. This may prove particularly useful with organic substrates that are toxic to cell growth and therefore prevent accumulation of biomass required for a successful growing cell reaction. Improvements to enzyme stability issues may lead to longer cellular productivity and would open the door to realizing valuable immobilized cell monooxygenase bio-reactors.

Our non-growing cell methodology provides reaction rates similar to other published examples of non-growing co-factor dependent oxidations performed in recombinant *E. coli*. Over-expression of a *Vibrio harveyi* flavin oxidoreductase for the

biodesulfurization of dibenzothiophene has yielded rates of 7.6  $\mu\text{mol}/\text{min}\cdot\text{g d.c.w}$  (Reichmuth *et al.*, 2000). The production of picolinic acid from 2-aminophenol has been performed via a dioxygenase system at a rate of 13  $\mu\text{mol}/\text{min}\cdot\text{g d.c.w}$  (He and Spain, 2000). In another example, xylene oxygenase was used to oxidize various aromatic hydrocarbon derivatives of toluene and xylene at rates of up to 18  $\mu\text{mol}/\text{min}\cdot\text{g d.c.w}$  for some substrates (Wubbolts *et al.*, 1994). Clearly these rates are dependent on specific substrate-enzyme pairs, but our efficiency is certainly within the same range as these examples.

We have examined whole cell total productivity limits with respect to several factors. Enzyme degradation appears to be the key factor and even though the origin of this process remains obscure, it appears to be simply a factor of enzyme stability under operational conditions. Oxygen, which is absolutely critical for catalysis, has also been suggested (Doig *et al.*, 2001) to promote the degradation process, although our data did not support this notion. FAD and NADPH co-factor levels are clearly sufficient for efficient catalysis. Substrate and product effects have been considered, but they are not significant under our conditions.

Analysis of the number of units of CHMO present in a given reaction also poses an interesting question. Typical crude cellular extracts show that CHMO is present at a starting concentration of 4500 U/L (U =  $\mu\text{mol}$  product/min). This extrapolates to a potential productivity of 270 mM per hour, forty times greater than typical observed rates. Clearly, something other than enzyme activity limits our whole cell bioconversions. One possibility, not investigated here, is the effect of pH. The reported pH optimum for *Acinetobacter* CHMO is 9.0 (Donoghue and Trudgill, 1976), while the

intracellular pH of *E. coli* is *ca.* 7.5 (Neiderhardt, 1987). It is therefore possible that this rate deficit could be due, at least in part, to the effect of pH. Whatever the responsible factor(s) is, identification could lead to significant productivity enhancements.

Future work required to improve on these successes can be divided into two areas: 1) analysis of the mechanism for enzyme loss and rate limits, and 2) manipulation of the host cell to enhance productivity.

Use of alternative BVMOs, or modified CHMO, may lead to improvements in enzyme stability and improved reaction rates. If sulfhydryl modification is the first step in CHMO enzyme degradation, then point mutations of *Acinetobacter* CHMO cysteine residues could lead to improved enzyme stability. Likewise, utilization of an alternate BVMO, that may lack a modifiable –SH group, might yield a simple solution to our enzyme degradation issue. Additionally, alternative BVMOs, one of which has demonstrated optimal activity at neutral pH (Trower *et al.*, 1985), may display better activity with respect to the enzyme titer in the recombinant system. Further optimization of enzyme titer during biomass production may also supply improvements to the non-growing cell technique.

Manipulation of the host cell might also provide gains in product yield. Oxygen is used for many processes within the cell. These demands may compete for available oxygen necessary for Baeyer-Villiger oxidation. Stark *et al.* have demonstrated a three to four fold increase in the activity of intracellular 2,4-dinitrotoluene dioxygenase when whole cells are doubly transformed with the gene for *Vitreoscilla* hemoglobin (Fish *et al.*, 2000). This bacterial hemoglobin traps oxygen thereby, in effect, increasing the intracellular concentration. This technique may be directly applicable to our

monooxygenase system. The BL21(DE3) host strain used in this work contains several protease knockouts. Even with these deletions it is possible that other specific proteases are responsible for CHMO degradation. Our results indicate that any such protein would have to be present prior to onset of stationary phase. Identification and deletion of any such proteins could allow for extended reaction times. In the event that CHMO loss is a passive process, rather than one initiated by a specific protease, enzyme levels could be maintained by starvation phase expression. During nitrogen starvation *E. coli* will express proteins designed to help cells survive during the stress of starvation (Siegele and Kolter, 1992). The Ntr regulon contains nitrogen starvation response genes. If CHMO was placed under control of the NtrC promoter, it might be possible to maintain a basal level of transcription under our non-growing cell conditions.

## CHAPTER 3 EXPERIMENTAL

### General

#### Materials

Cyclohexanone,  $\epsilon$ -caprolactone, and Amberlite XAD-4 resin were obtained from Sigma-Aldrich and used as received. Media components, ampicillin, and IPTG were purchased from Fisher Scientific and were prepared as described below.

#### Gas Chromatography

GC analysis of substrate and product was conducted with a Hewlett-Packard Model 5890 GC/FID fitted with a 30 m DB-17 column or a Perkin-Elmer 8500 GC/FID equipped with a 30 m HP-5 column. The standard temperature program used consisted an initial time and temperature of 2 minutes and 50° C, a 10° C per minute temperature gradient, and a final time and temperature of 5 minutes and 180° C for a total analysis time of 20 minutes.

Samples were prepared for analysis by vigorously mixing 200  $\mu$ L of the aqueous reaction media with 600  $\mu$ L ethyl acetate containing 1 mM methyl benzoate (internal standard) followed by removal of the organic layer. This step was repeated with a second volume of ethyl acetate mixture and the two organic phases were combined. Optimal consistency was obtained by allowing each extraction to equilibrate 10 minutes prior to removal of the organic layer.

Standard curves for cyclohexanone,  $\epsilon$ -caprolactone, and ethyl-(*S*)-3-hydroxybutyrate were prepared by making aqueous mixtures of each at various concentrations and extracting as described above. Results were linear up to 100 mM. Ethyl acetoacetate decomposed during GC analysis and was therefore not quantified.

### **Glucose Assay**

Glucose concentrations were determined offline using the Trinder reagent kit commercially available from Sigma. Five microliter aliquots of water (blank), 0.4 g/L glucose (standard) and the unknown were added to three separate 1 mL aliquots of reconstituted Trinder reagent. After gentle inversion, the tubes containing each were incubated in a dry bath at 37° C for 15 minutes. Immediately following incubation, the absorbance at 505 nm was measured. Unknown concentrations were determined by reference to the standard absorbance.

### **Media Preparation**

Luria-Bertani (LB) medium used for cell growth was prepared by combining 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride followed by autoclave sterilization. When used for plates, media was solidified using 15 g/L agar. When specified for growth, glucose was added to 4 g/L in LB media by addition of 20% w/w aqueous glucose that had been autoclaved separately.

M9 minimal medium was prepared by autoclaving a mixture of  $\text{Na}_2\text{HPO}_4 \cdot 7 \text{ H}_2\text{O}$  (12.8 g/L),  $\text{KH}_2\text{PO}_4$  (3 g/L),  $\text{NaCl}$  (0.5 g/L), and  $\text{NH}_4\text{Cl}$  (1 g/L). Separately sterilized aliquots of  $\text{MgSO}_4$ ,  $\text{CaCl}_2$ , and 20% glucose were then added to bring to final volume with concentrations of 2 mM, 0.1 mM, and 4 g/L respectively. Ammonium chloride was omitted when used for non-growing cell reactions.

### Cell Culturing

*E. coli* BL21(DE3)(pMM4) was streaked fresh weekly, from 15% glycerol stock stored at -80° C, onto LB plates supplemented with 200 mg/L ampicillin, and incubated overnight at 37° C until colonies formed. Single colonies were then used to prepare inocula for cell growth.

When necessary, a fresh stock of BL21(DE3)(pMM4) was prepared by transformation of fresh BL21(DE3) (Novagen) with pMM4 plasmid via electroporation. Construction of pMM4 kept in frozen stock has been described previously (Chen *et al.*, 1999).

### Biotransformation with Growing Cells

A single colony of BL21(DE3)(pMM4) was used to inoculate 50 mL of the desired growth medium supplemented with 200 mg/L ampicillin and the flask was shaken overnight at 37° C. Thirty milliliters of the culture was then added to 3 L of the same medium containing 200 mg/L ampicillin and 1 mL anti-foam AF-204 (Sigma). The culture was then grown in a Braun Biostat E at a temperature of 32° C, agitation at 300 rpm and aeration at a minimum of 0.3 v.v.m. (1 v.v.m. maximum). Upon reaching the desired OD<sub>600</sub> (2.0 in LB or 1.0 in LB + glucose), IPTG was added to a concentration of 0.1 mM followed by substrate 20 minutes later. (When reactions were performed in M9 medium, IPTG was added at the time of inoculation and substrate was added at OD<sub>600</sub> = 1.0). Substrate was injected as a single bolus addition to a final concentration of 10 mM or, in the case of the glucose fed scenario, as a 15 mM bolus followed by pump feed at an empirically determined rate. The ketone substrate was pumped using a Beckmann 110 HPLC solvent delivery module with accuracy limit of 0.01 mL/min. Reaction progress

was monitored by GC as described above. When used, glucose steady state was maintained by a feed of 20% glucose through sterile in-line filter based on the results of the glucose assay previously described. Glucose feed was controlled in 1 mL/hr increments (typical demand is 10 mL/hr).

#### **Biotransformation with Non-growing Cells**

A single colony of BL21(DE3)(pMM4) was used to inoculate 2 x 50 mL of LB media supplemented with 200 mg/L ampicillin and the culture was shaken overnight at 37° C. The entire 100 mL of pre-culture was added to 10 L of LB media containing 4 g/L glucose, 200 mg/L ampicillin and 2 ml anti-foam AF-204 (Sigma) in a Virtis 43-100 fermenter at a temperature of 37° C, agitation at 700 rpm and aeration 1.1 v.v.m. Upon reaching  $OD_{600} \sim 1.0$ , IPTG was added to a final concentration of 0.1 mM. At six hours, or when  $\Delta OD_{600} < 1$  per hour, the broth was transferred to 2 L flasks and placed on ice until centrifugation and consolidation of the total pellet was complete. The cells were then re-suspended in 200 mL of the non-growing cell media described above and supplemented with 2.5 mg/L riboflavin. The resulting slurry was generally used within 24 hours, but has been shown to maintain activity for up to five days.

For the biotransformation, the cell slurry was added to 3 L of non-growing cell media, supplemented with 1.0 mg/L riboflavin, to a final  $OD_{600}$  of approximately 15. Substrate was injected as a bolus addition to a final concentration of 30 mM followed by two more 30 mM bolus additions three hours apart. Alternatively, the ketone was fed by pump at an empirically determined rate (to maintain a near steady-state level) after the initial 30 mM bolus. Reaction progress was then monitored by GC as described above. The glucose concentration was also maintained at a near steady-state by feeding 20%

glucose solution by a pump at a rate based on the results of the glucose assay previously described. Typical glucose demand was 10 mL/hr. Sterility was not maintained when conducting operations under non-growing conditions.

### **Enzyme Assays**

Enzyme assays were performed by collecting a 100 mL aliquot from the desired reaction vessel, then centrifuging at 5000 rpm for 10 minutes at 4°C and washing the pellet with 10 mL of 60 mM phosphate buffer, pH 7.0. Washed cells were centrifuged at 5000 rpm for 10 minutes and re-suspended in 2.5-3 mL of the same buffer before being broken by a French pressure cell, then the soluble fraction was isolated after centrifugation at 13,500 rpm for 10 minutes. Crude protein preparations were stored up to 2 days at 4° C without any observed loss of CHMO activity.

Determination of CHMO activity in crude extracts was carried out according to Donoghue (Donoghue and Trudgill, 1976). Crude extracts were diluted 1:100 with the previously described phosphate buffer. Assays were conducted at 30° C in a 1 cm path quartz cuvette containing 80 mM glycine/NaOH buffer, pH 9.0, 80  $\mu$ M NADPH, 15  $\mu$ M FAD, and 10  $\mu$ L of diluted crude protein (crude extracts range from 10 to 20 mg/mL). After measuring the rate of absorbance loss at 340 nm with no ketone added, cyclohexanone was added to a final concentration of 0.6 mM and the consumption of NADPH was measured by a decrease in absorbance at 340 nm. Total protein estimation was done via the Bradford method (Bradford, 1976) and compared to freshly prepared standard concentrations of bovine serum albumin. From these data, specific activities were determined and defined as  $\mu$ mol of  $\epsilon$ -caprolactone produced per minute per mg total soluble cellular protein. Qualitative determinations of enzyme levels over time was

performed by loading equal amounts of crude cell proteins on a 10% SDS-PAGE gel followed by electrophoretic separation and Coumassie Blue staining for visualization.

### Cofactor Assays

Cycling assays for determination of nicotinamides were based on the method published by Bernofsky and Swan in 1973. Actual protocols used were identical to the methods developed by Gibon and Larher in 1997 for determination of nicotinamides in plant tissues. This method yielded values consistent with those published in the literature (Lilius *et al.*, 1979). Enzymes used for these assays were purchased from Sigma and were specifically sold as preparations containing minimal exogenous nicotinamides.

Nicotinamides were extracted directly from reaction media. Oxidized forms ( $\text{NAD}^+$ ,  $\text{NADP}^+$ ) were extracted by acidifying 0.5 mL aliquots of cells with 0.25 mL of 0.3 N HCl. Reduced forms (NADH, NADPH) were extracted by addition of 0.25 mL of 0.3 N KOH and 10 mg CuCl to a 0.5 mL cell suspension. Both extractions were vortexed, heated at 60° C for 7 minutes and then frozen at -20° C until ready to assay. Thawed extractions were neutralized with either acid or base and centrifuged for 15 minutes. The supernatants were then removed to obtain the crude co-factor extract.

An assay mixture was freshly prepared containing 0.2 M Tricine/NaOH, pH 8.0, 8 mM EDTA, 332  $\mu\text{M}$  phenazine ethosulphate (PES), 840  $\mu\text{M}$  3-(4,5-dimethylthiazoyl-2)-2,5-diphenyltetrazolium bromide (MTT), and either 1 M ethanol or 5 mM glucose-6-phosphate. After preparation, these solutions were kept in the dark. Crude co-factor samples were added to separate tubes in volumes of 50  $\mu\text{L}$  for each  $\text{NADP}^+$  and NADPH, 15  $\mu\text{L}$  for  $\text{NAD}^+$ , 100  $\mu\text{L}$  for NADH, and volumes of known standards containing up to 200 pmol of co-factor were brought to 400  $\mu\text{L}$  volume with 0.1 M NaCl. To each, 0.5 ml

of the appropriate assay mixture (EtOH for NAD, G-6-P for NADP) was added and allowed to incubate 5 minutes in the dark at 37° C. To these tubes, 10 units of alcohol dehydrogenase for NAD assay, and 0.27 units of glucose 6-phosphate dehydrogenase for NADP assay were added, gently inverted, and allowed to cycle for at 37° C in darkness. After 40 minutes, all tubes were quenched with 0.5 ml saturated NaCl. Tubes were then centrifuged for 10 minutes at 4° C and the supernatant was carefully removed and discarded. The undisturbed precipitate was then dissolved in ethanol and absorbance was measured at 570 nm. Concentrations of each form of nicotinamide were then calculated in nmoles per mg of dry cell weight by comparison to the freshly prepared standard curve.

#### **Product Recovery**

Product recovery was carried out by passing the biotransformation mixture over the XAD 4 column containing 200 grams of resin. The column was then washed with 500 ml of acetone followed by 500 ml of water. The acetone fraction was concentrated by rotary evaporator under aspiration at 32° C, leaving a small aqueous residue (50 mL). Before passing the reaction mixture over the column again, the resin was washed with 2 L of fresh water to remove any residual acetone. This step was repeated with fresh acetone and the original 500 mL portion of water three more times. All of the concentrated acetone extracts and the 500 ml aqueous wash were combined and  $\epsilon$ -caprolactone was recovered by continuous extraction with 500 mL of ethyl acetate for 24 hours. Ethyl acetate was removed by rotary evaporator and the product was purified by distillation (bp 128° C, water aspirator).

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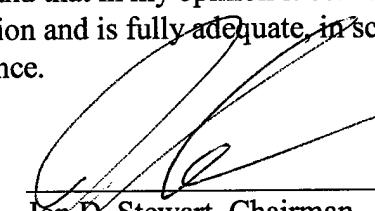
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

  
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Jon D. Stewart, Chairman  
Associate Professor of Chemistry

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

  
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This thesis was submitted to the Graduate Faculty of the Department of Chemistry in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Master of Science.

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